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THE JOURNAL OF  
EXPERIMENTAL MEDICINE



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# THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY  
SIMON FLEXNER, M.D.

VOLUME THIRTY-THIRD  
WITH ONE HUNDRED AND EIGHT PLATES AND  
ONE HUNDRED AND SEVEN FIGURES  
IN THE TEXT



166957-  
9/11/21

NEW YORK  
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH  
1921

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WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A.

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# THE RETICULAR MATERIAL OF DEVELOPING BLOOD CELLS.

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PLATE 1.

(Received for publication, September 14, 1920.)

The object of this paper is to determine the appearance of the reticular material of normal blood cells with a view to preparing the way for further experimental studies, using it as an indicator of cell activity and cell injury. The extensive literature on the subject of this material has already been reviewed in detail by Holmgren (1901-02), Benda (1914), Duesberg (1914), and Cajal (1914). Very little has been done on the blood.

I have used the bone marrow of guinea pigs, applying the osmic acid method of Kopsch (1902),<sup>1</sup> the uranium and silver nitrate method of Cajal (1912),<sup>2</sup> and the methods of fixation recommended by Bensley (1910).<sup>3</sup> At the same time, the observations have been controlled by the examination of unstained and supravitaly stained living cells by direct and oblique illumination.

Sinigaglia (1910) was apparently the first to discover a circumnuclear, net-like structure in the red blood cells of young amphibia, and von Berenberg-Gossler (1912)<sup>4</sup> has recorded a similar but eccentrically placed network in the blood cells of chick embryos. Fañanás (1912),<sup>5</sup> with the aid of Cajal's silver and uranium nitrate method, has attempted to determine the changes which this network undergoes during the development of red blood cells. He has found that with the appearance of hemoglobin it undergoes regressive changes, becoming condensed and finally disappearing completely, but he does not claim that there is any functional relation between its disappearance and the formation of hemoglobin.

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<sup>1</sup> Kopsch (1902), p. 929.

<sup>2</sup> Cajal (1912), p. 211.

<sup>3</sup> Bensley (1910), p. 192.

<sup>4</sup> von Berenberg-Gossler (1912), p. 62.

<sup>5</sup> Fañanás (1912), p. 251.

*Results Obtained with Erythroblasts.*

Though I have been unable to detect any trace of the existence of reticular material in living red blood cells, I have produced a blackened network in the cytoplasm of erythroblasts by the methods of Kopsch and Cajal. Of the two methods the prolonged treatment with osmic acid, as advised by Kopsch, is in my opinion subject to the least criticism because it is relatively simple and does not distort the cells or destroy the specific granulations. Figs. 4 to 12 illustrate the irregularity and variability of the reticular material in erythroblasts.

Like von Berenberg-Gossler, I find that it is usually eccentrically placed and rarely if ever circumnuclear. In my preparations it is generally compact, varying, however, in size as well as in the coarseness of its meshes. Frequently in neighboring cells no trace of it can be seen. In the cells which show it there are usually some blackened granules of variable size. It almost seems as though stages in the formation of the network can be distinguished through the alignment and confluence of these granules, or that the granules are produced through the disintegration of the network. As the erythroblasts develop, the reticular material becomes smaller in amount and finally disappears in the mature erythrocytes. It is impossible to say how close is the parallelism between these changes and the formation of hemoglobin, because my preparations do not permit an accurate determination of the amount of hemoglobin.

Cajal's method brings to light a similar network, but unfortunately the preservation of the cells is so poor that it is often difficult even to identify them. Erythroblasts prepared by this method are shown in Figs. 14, 15, 18, and 20. In spite of their larger size and greater complexity, these networks resemble closely those in the Kopsch preparations described above. Similar blackened granules are also to be seen which perhaps bear the same relation to the network. Fig. 18 is suggestive in this connection because the blackened granules seem to be embedded in a more lightly colored material which forms the strands of the network.

The relation, however, between this blackened reticular material and the canalicular apparatus which may be demonstrated in the erythroblasts by fixation in a mixture of formalin and potassium

bichromate, as recommended by Bensley (1910), is not so clear. It is true that this system of clear canals is usually eccentrically placed to one side of the nucleus (Figs. 22, 23, and 25), that clear droplets suggestive of the blackened granules may often be discerned near it, and that it disappears as differentiation goes on, so that no trace of it can be found in mature erythrocytes (Fig. 28); but, on the other hand, the clear canals are more sharply defined (Fig. 27) and are often of greater girth (Fig. 30). They tend also to be more pointed and certainly look more like fixation artifacts, or better, greatly distorted pictures of some actually existing material, particularly when they are very pronounced as in Figs. 26, 31, and 36.

Although I have employed a large variety of mixtures and their individual ingredients in different concentrations, I have not been successful in attempts to determine what factor or factors in fixation are responsible for the appearance of these canals, which cannot be seen clearly in the living cell. It is difficult, if not impossible, to alter the caliber of the canals experimentally. Duesberg (1920)<sup>6</sup> postulates the existence of two distinct kinds of canalicular apparatus (trophospongium) one of which is identical with the reticular material (apparatus of Golgi) and the other is not. He writes as follows:

"I came to the conclusion . . . that two categories of cells should be distinguished: The neurones and non-nervous cells with a localized trophospongium on the one side, and the non-nervous cells with a diffuse trophospongium on the other. As to the latter, the identity of both formations can be rejected without further discussion; for, while the trophospongium extends all over the cytoplasm, the apparatus of Golgi is localized at one pole of the nucleus. As to the former, both formations appear to me identical."

The expression "identical" is one which I should hesitate to apply, as it cannot be interpreted to mean anything less than similarity in all respects. Obviously, the blackened apparatus of Golgi is not identical with a system of clear canals brought to light by a totally different method. One is a silver deposit, the other a portion of the cytoplasm devoid of silver which can only be stained with great difficulty. If we refer to two cells even of the same category or

<sup>6</sup> Duesberg (1920), p. 75.

to minute portions of them as being identical we are overlooking the fact that living material is constantly changing both qualitatively and quantitatively and that it is these changes which make life possible.

According to Duesberg's theory there are two kinds of trophospongium which are distinguished solely by the fact that the trophospongium is either localized or diffuse. There are no other differential characteristics. As a matter of fact, all the other known properties of the localized and diffuse trophospongium appear to be similar. Duesberg does not give sufficient consideration to the vagaries of technique. It is conceivable that the diffuse canals called trophospongium and the localized apparatus of Golgi, though quite different in appearance in fixed tissues, may have been produced through the action of diametrically opposed methods of technique upon somewhat similar material. Within the compass of a single cell whose cytoplasm is fluid and in more or less constant motion spacial differences are not of very great moment. A comparison of Figs. 22, 23, 25, 26, and 30 shows the remarkable variations in the topography of the trophospongium which may be seen in cells of the same type treated in the same way.

#### *Results Obtained with Leucocytes.*

Von Bergen (1904),<sup>7</sup> Kolster (1913), and others have alluded to the occurrence of reticular material in granular leucocytes. Here, as in the case of the erythroblasts, the methods of Kopsch and Cajal reveal what cannot be seen by the most painstaking observation of living cells. By the method of Kopsch a few scattered granules are blackened in the amphophils and eosinophils (Figs. 1 and 2), while nothing is brought to light in the basophils (Fig. 3). The lack of definite grouping on the part of these granules points to the conclusion that they may have nothing to do with the reticular material. Fortunately, Cajal's method gives more definite results (Figs. 13, 16, 17, 19, and 21). It will at once be remarked that the blackened networks are much smaller in proportion to the size of the cells than in erythroblasts. They are also more condensed, with closer meshes, and are

<sup>7</sup> von Bergen (1904), p. 542.



almost invariably situated in the concavity of the nucleus near the centrosome. Blackened granules, like those in the erythroblasts, are not apparent in the amphophilic leucocytes shown in Figs. 13, 16, and 17. This may mean that all the material has been used up in the formation of the networks.

Granular leucocytes in preparations made by fixation in formalin and potassium bichromate are depicted in Figs. 24, 29, and 32 to 35. In Fig. 24 the canals are shown in an amphophil. They are much larger than the blackened reticular material and are not restricted to the region of the nuclear concavity. In Fig. 29 they are just as large and conspicuous as in certain erythroblasts; *i.e.*, Figs. 26 and 30. Other myelocytes and leucocytes fail to show any trace of the canalicular apparatus (Figs. 32 to 35). Considerable variability is apparent even in adjacent cells in the same section.

#### *Results Obtained with Lymphocytes.*

I have not been successful in finding any trace of the presence of a reticular or canalicular apparatus in living cells of the lymphocytic series. In the Kopsch and Cajal preparations of the bone marrow it is only possible to distinguish the lymphoblasts from the erythroblasts through the absence of hemoglobin, and then not with certainty, the nuclear characteristics being indistinct. I have found, however, in common with other workers, that the lymphocytes inhabiting other tissues, such as the thyroid, show a very definite blackened network at one side of the nucleus when treated by Cajal's method. In this respect they resemble closely the erythroblast illustrated in Fig. 10.

#### DISCUSSION.

There can be no doubt that these observations indicate the existence of some material in the cytoplasm of developing red blood cells, leucocytes, and lymphocytes which reacts in a more or less characteristic way to two quite different methods of technique. Since investigators have also found that a large number of nerve cells, gland cells, and epithelial cells, in fact almost all the active cells of the body, react in much the same way when treated by these methods, this substance, or substances very closely related to it, must be widely

distributed in living protoplasm, and of fundamental importance. This material tends to exhibit the following properties. (1) Under certain conditions, which have not yet been fully determined, it may be blackened by treatment with osmic acid and silver nitrate. It may also be frequently stained with resorcin-fuchsin after fixation in trichloroacetic acid. Though I have obtained satisfactory preparations by this method in other tissues (brain, pancreas, etc.) the blood cells have thus far proved refractory. (2) The material is arranged in a network of anastomosing strands, the form of which is to some extent characteristic of different cell types. (3) This network is broken up on cell division and is distributed in approximately equal amounts to the two daughter cells. It undergoes definite and progressive changes during cell differentiation. (4) In secretory cells with pronounced polarity, such as the acinus cells of the pancreas, it is invariably found in the region of the cytoplasm between the nucleus and the distal border. When the material is distributed in the form of a circumnuclear network it usually occupies an intermediate position, leaving a zone of clear cytoplasm about the nucleus and beneath the cell wall. (5) It is often, though not always, found in close association with the centrosome which is presumably a center of unusually high or low cytoplasmic activity.

Following the discovery by Golgi of reticular material in nerve cells, claims were made, which have not since been substantiated, that this material is composed of easily recognizable cytoplasmic constituents like the mitochondria and the chromidial substance. Recent work, however, tends to show that it is a constituent of the ground substance which in living cells appears to be homogeneous when viewed with the best apochromatic lenses. This homogeneity is, of course, apparent, not real, for true homogeneity would be incompatible with chemical activity. Suitable methods for the detection of this reticular material in living cells are at present lacking. We have no reason to suppose that the form of the reticular material in living protoplasm is very different from that seen in fixed preparations. It is probable, however, that in the living condition its outlines are less distinct and that it merges gradually into the remainder of the cytoplasm. With fixation a certain amount of condensation and shrinkage occurs so that this reticular material would

occupy a rather larger area of the cytoplasm in the living cell than in fixed preparations. The phenomena of ameboid motion, phagocytosis, and the freedom of motion of certain intracellular granules are incompatible with the existence of a rigid network within the cell. Moreover, M. R. Lewis and W. H. Lewis (1914-15) have repeatedly studied cultures of living connective tissue cells, yet they have never recorded the presence of a semisolid network of material, although Deineka (1912)<sup>8</sup> experienced no difficulty in bringing a network to light in connective tissue cells by his silver method. It is interesting also to note that Chambers (1915) has actually dissected cells in which the existence of this network of material has been demonstrated by the methods of Kopsch and Cajal without remarking upon the occurrence of any kind of framework of resistant nature. All the information at hand suggests that, though the material is restricted to certain cytoplasmic areas, it is nevertheless of watery consistency, being even more fluid than the general ground substance, and is probably changing its form continually as it plays its obscure part in the activities of the cell, for no two networks as seen in fixed preparations of blood cells, or any other kind of cell, are exactly alike.

If this idea regarding the fluidity of the material is correct we must be dealing with a system of intracellular spaces or canals as claimed by Bensley. The walls of the canals appear to be extremely thin. It has never been possible to stain them in fixed preparations. In all probability they are merely surfaces of contact between fluids of different character. Slight changes in the reticular material or in the surrounding cytoplasm would certainly modify the visibility of the surfaces. It seems to me also that in living protoplasm the reticular material may to some extent intermingle with the surrounding cytoplasm instead of being sharply separated from it. Since its refractive index is apparently the same as that of the cytoplasm it could not be expected to be visible in the living cell until selective methods for its coloration have been devised. Bensley (1911-12),<sup>9</sup> however, has been able to observe a system of clear canals in islet cells of the pancreas stained supravitaly with neutral red. The canals show up as clear spaces in a cytoplasm otherwise crowded with

<sup>8</sup> Deineka (1912), pp. 303, 306.

<sup>9</sup> Bensley (1911-12), p. 365.

granules. On one occasion I observed a similar phenomenon in nerve cells (1912-13)<sup>10</sup> but all my efforts to discern this reticular material in blood cells and in other cells of the body have thus far been unavailing. In both these cases some time elapsed between the removal of the cells from the animal and the detection of the canals. Their appearance may, therefore, be due, as already intimated, to some change in the reticular material or in the cytoplasm, or in both. The fluidity and lability of the material would make it particularly susceptible to the change in environment.

Though the question of terminology is a minor one the least criticism will be caused if this peculiar substance, or mixture of substances, is called reticular material, instead of reticular apparatus, until more definite information is obtained about it. The idea of a distinct system of canals is not sustained, and speaking of it as a cell organ suggests that it is a more or less complex unit built up of specialized components each with a definite function, like the constituent tissues of the bodily organs, in favor of which there is as yet insufficient evidence. Study from the standpoint of microchemistry would throw further light on this subject.

The wide distribution of this reticular material in the three categories of blood cells as well as in active cells of almost all kinds, and the reasonableness of Bensley's (1910)<sup>11</sup> hypothesis that the canalicular apparatus is "the physiologic and morphologic equivalent of the vacuolar system of the plant cell," make it apparent that this is a cytoplasmic constituent of great importance.

#### SUMMARY.

Erythroblasts, leucocytes, and lymphocytes resemble other cells of the body in containing a restricted area of fluidity in their cytoplasm. In special preparations this fluid appears in the form of a more or less complicated reticulum which seems to be continually but slowly changing in shape. For convenience the fluid may provisionally be referred to as reticular material, emphasis being laid on its composition rather than its form. As a working hypothesis it is safe to

<sup>10</sup> Cowdry (1912-13), p. 490.

<sup>11</sup> Bensley (1910), p. 193.

assume that the chemical and physical properties of this material vary in cells of different kinds as well as in different stages in the activity of the same cell. The conclusion that it is "identical" in different cells, because the present crude methods of technique reveal no fundamental differences, would be as incorrect as the statement that the serum of different animals is identical, because no difference is observed on microscopic examination.

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## EXPLANATION OF PLATE 1.

All the figures have been drawn with a Zeiss objective 1.5 mm., compensating ocular 8, and camera lucida. Magnification,  $\times 2,200$ .

FIGS. 1 to 12. Cells from the bone marrow of an adult male guinea pig fixed in 2 per cent osmic acid for 15 days for the demonstration of the reticular apparatus after the method of Kopsch.

FIGS. 13 to 21. Cells from the bone marrow of a male guinea pig of 240 gm. prepared by the uranium nitrate method of Cajal with slight modifications.

FIGS. 22 to 36. Cells from the bone marrow of the same guinea pig fixed in Kopsch's formalin-bichromate mixture and stained with fuchsin and methyl green.

FIG. 1. Amphophil leucocyte without any trace of a blackened network. A few granules are present which are blackened with osmic acid; the others are of a dull gray tone.

FIG. 2. Eosinophil leucocyte with no indication of a blackened network. It contains a series of smaller granules which are intensely blackened and other larger spherical ones of a gray color.

FIG. 3. Basophil leucocyte without either network or blackened granules.

FIG. 4. Large erythroblast with blackened granules exhibiting a tendency toward network formation.

FIG. 5. Erythroblast with a rather more definite network at one side of the nucleus.

FIG. 6. Erythroblast containing only three spherical blackened granules of variable size.

FIG. 7. Erythroblast with granules of more irregular shape.

FIG. 8. Erythroblast with apparently clear cytoplasm.

FIG. 9. Erythroblast with an almost completely blackened network, the individual strands of which may be followed by careful focusing. The cytoplasm contains also some blackened granules.

FIG. 10. Erythroblast with a blackened network of much closer mesh. No blackened granules are to be seen in the cytoplasm.

FIG. 11. Erythroblast with a large open network and cytoplasmic granules.

FIG. 12. Erythroblast with a blackened network of which the strands are noticeably more slender.

FIG. 13. Polymorphonuclear amphophil leucocyte with several large blackened granules in the concavity of the nucleus in the region where the centrosome is located.

FIG. 14. Large erythroblast with a complete network at one pole of the nucleus.

FIG. 15. Large erythroblast with an extensive and very intricate network and some cytoplasmic granulation.

FIG. 16. Polymorphonuclear amphophil leucocyte with a very simple network in the concavity of the nucleus.

FIG. 17. Polymorphonuclear amphophil leucocyte with slightly more complicated network in the same location.

FIG. 18. Large cell, possibly an erythroblast, showing the formation of a network by the alignment of discrete granules.

FIG. 19. Amphophil myelocyte with network formation in the nuclear concavity.

FIG. 20. Erythroblast with very circumscribed, closely meshed network.

FIG. 21. Coarsely granular leucocyte, probably eosinophilic, showing likewise network formation in the concavity of the nucleus.

FIG. 22. Erythroblast containing three irregular clear spaces (negative demonstration).

FIG. 23. Erythroblast with the clear canals forming a complicated coil.

FIG. 24. Amphophil polymorphonuclear leucocyte with clear canals in the peripheral cytoplasm as well as in the concavity of the nucleus.

FIG. 25. Erythroblast with clear canals and vacuoles distributed throughout the entire cytoplasmic area.

FIG. 26. Large cell, probably also an erythroblast, showing the extreme of canalicular and vacuolar formation as well as some crenation. The canals and vacuoles are of large caliber.

FIG. 27. Erythroblast containing clear canals of very fine diameter.

FIG. 28. Erythrocyte devoid of any trace of canalicular formation.

FIG. 29. Large myelocyte containing a very complicated system of clear canals.

FIG. 30. Erythroblast with canals of maximum caliber. The forked ones are especially characteristic.

FIG. 31. Erythroblast containing a peculiar clear circle.

FIG. 32. Basophil polymorphonuclear leucocyte without any indication of canalicular formation.

FIG. 33. Basophil myelocyte likewise without visible canalicular apparatus.

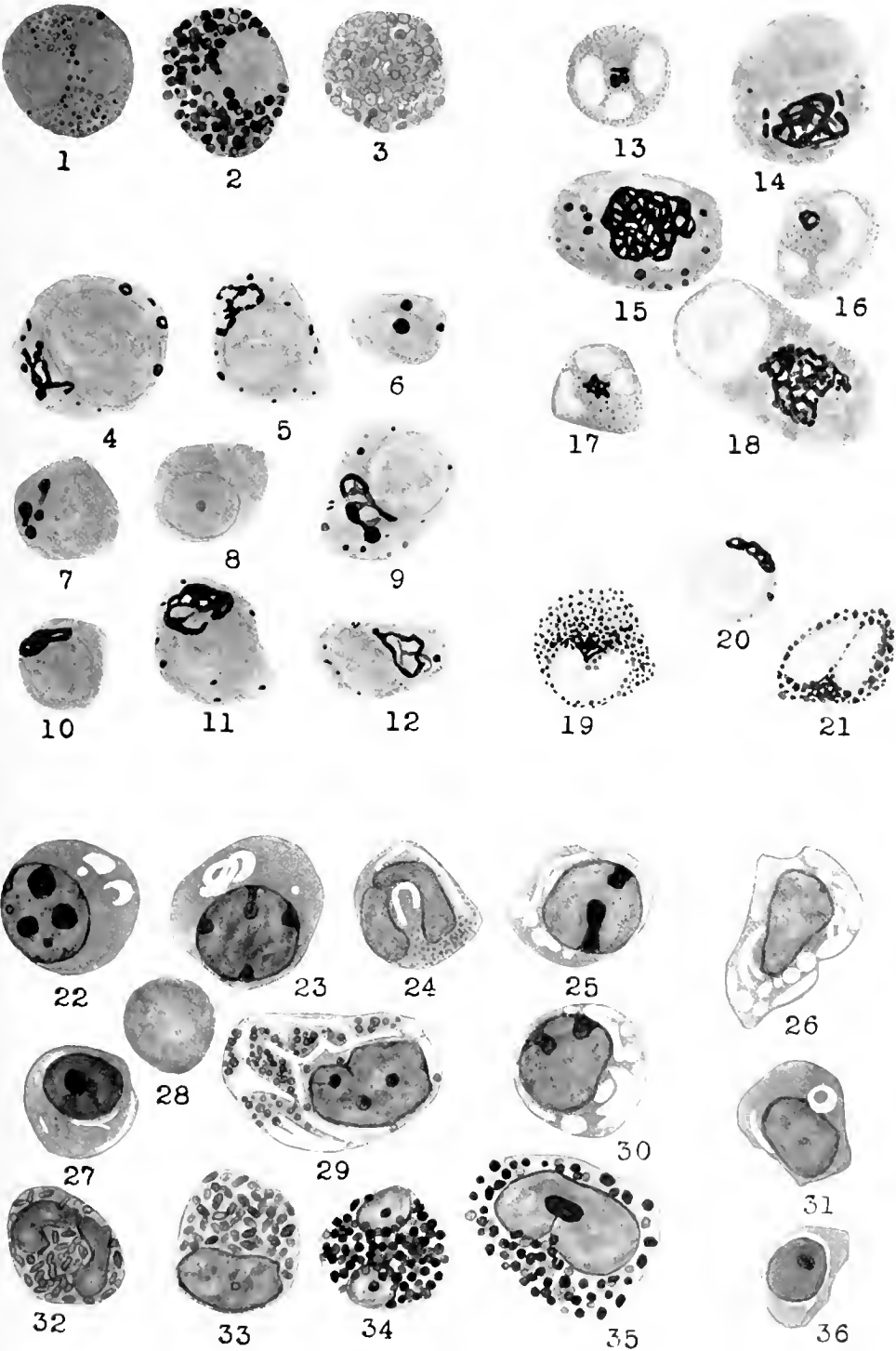
FIG. 34. Eosinophil leucocyte in the same condition.

FIG. 35. Eosinophil myelocyte without any sign of canalicular apparatus.

FIG. 36. Erythroblast with clear clefts almost surrounding the nucleus.







(Cowdry: Developing blood cells.)



## STREPTOCOCCI OCCURRING IN SOUR MILK.

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(Received for publication, June 24, 1920.)

Considerable confusion seems to exist regarding the specific cultural characters of the group of organisms which sour milk. Many have noted the presence of enormous numbers of streptococcic forms in sour milk. The morphology of such bacteria has been studied in detail by Grotenfeld,<sup>1</sup> Günther and Thierfelder,<sup>2</sup> Kruse,<sup>3</sup> and others. Kruse has given the name of *Streptococcus lacticus* to the organism most frequently found and called attention to its resemblance to the pneumococcus. Many, however, disagree with Kruse and place it in the genus *Bacterium*; such names as *Bacterium lacticus*, *Bacterium g ntheri*, and *Bacillus lactis acidi* are frequently met with.

In addition to the confusion in nomenclature, one frequently finds reference to the occurrence of *Streptococcus lacticus* in the udder, feces, and saliva, or on the skin of cows. Furthermore, many believe that the milk-souring type is indistinguishable in most respects from the pyogenic type.

Ruediger<sup>4</sup> studied streptococci isolated from milk freshly drawn into milk pails and sterile bottles. Of 32 strains grown in blood agar plates, 8 produced considerable hemolysis about the colonies, 14 were faintly hemolytic, 9 produced green zones, and 1 was non-hemolytic. All the organisms were cocci arranged in chains; rod-like elements were not observed. Ruediger considered that the eight hemolyzing strains were *Streptococcus pyogenes* and the other faintly hemolyzing and green-producing organisms belonged to the *Streptococcus lacticus* group. Further evidence to warrant this assumption is not submitted.

Heinemann<sup>5</sup> states that the group comprises two species,—a bacterium, *B. aerogenes* var. *lacticus* and *Streptococcus lacticus*. It is stated that *Streptococcus lacticus* agrees in morphological and cultural characters with the human pathogenic, sewage and fecal streptococci. He believes that the milk-souring streptococci can be detected on the external surfaces and in the feces of cows as well as

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<sup>1</sup> Grotenfeld, G., *Fortschr. Med.*, 1889, vii, 121.

<sup>2</sup> G nther, C., and Thierfelder, H., *Arch. Hyg.*, 1895, xxv, 164.

<sup>3</sup> Kruse, *Centr. Bakt., He Abl., Orig.*, 1903, xxxiv, 737.

<sup>4</sup> Ruediger, G. F., *Am. J. Pub. Health*, 1912, ii, 107.

<sup>5</sup> Heinemann, P. G., *J. Infect. Dis.*, 1906, iii, 173.

in the milk during all stages of handling. In a later paper Heinemann<sup>6</sup> published the results of some experiments with two strains of streptococci, one isolated from certified milk, the other from ice-cream. By the passage of these cultures through a series of rabbits and guinea pigs their virulence was increased. In addition, the organisms grew feebly. They produced much less acid in dextrose and lost the power to ferment saccharose, mannitol, and salicin.

Conn, Esten, and Stocking<sup>7</sup> described *Streptococcus lacticus* as a short chained, Gram-positive coccus, which grew on agar as an exceedingly delicate film. Gelatin colonies were characteristic but the medium was not liquefied. Dextrose, lactose, and saccharose were fermented without gas formation. Milk was coagulated. They found these organisms in great numbers in sour milk and state that they frequently made up 99 per cent of the flora.

Shippen<sup>8</sup> in studying the microorganisms of milk in Baltimore referred to *Streptococcus lacticus* as *Bacterium g ntheri*. It was the most common type observed by him in sour milk but its presence was also noted in fresh (unsoured) milk. Like Kruse, he stated that the forms varied from short slender rods to distinct coccus-like forms. Many strains closely resembled streptococci when grown in liquid media, but search always revealed some rod-like forms. In these cultures the elongated elements predominated but cocci could also be found. He considers that the sour milk organisms differ from *Streptococcus pyogenes* in several respects; namely, in form, in their inability to hemolyze, their ability to grow at lower temperatures, their lack of pathogenicity for laboratory animals, and their high thermal death-point (65–70°C.).

McGuire<sup>9</sup> considers that *Streptococcus lacticus* is a normal inhabitant of cow feces. Apparently the species was identified largely by morphology and the points noted by Shippen.

Weigmann<sup>10</sup> divides the milk streptococci into two groups: the *Streptococcus mastitidis* Guillebeau group characterized by the coagulation of milk and the formation of gas from carbohydrates; and the lactic acid group, *Streptococcus lacticus*, or *Streptococcus g ntheri*. The latter class fails to produce gas. It is surprising to note the frequency with which the mastitis streptococci are referred to as gas-producing organisms throughout the literature.

Sherman and Albus<sup>11</sup> have shown that udder streptococci differ from those found in sour milk. They isolated and studied 50 strains from sour milk and as

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<sup>6</sup> Heinemann, P. G., *J. Infect. Dis.*, 1915, xvi, 221.

<sup>7</sup> Conn, H. W., Esten, W. M., and Stocking, W. A., *Rep. Storrs Agric. Exp. Station*, 1906, 91.

<sup>8</sup> Shippen, L. P., *Bull. Johns Hopkins Hosp.*, 1914, xxv, 122.

<sup>9</sup> McGuire, P. F., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 386.

<sup>10</sup> Weigmann, H., in Sommerfeld, P., *Handbuch der Milchkunde*, Wiesbaden, 1909, 328.

<sup>11</sup> Sherman, J. M., and Albus, W. R., *J. Bact.*, 1918, iii, 153.

many from milk drawn directly from the udder into sterile bottles. The two classes of organisms were studied in groups. It was found that the individual elements of the sour milk type were elongated and grouped most frequently in pairs, but chain formation was common. The udder streptococci were spherical and grew as chains. The action of the two groups in the various media was noted. Thus 40 per cent of the lactic acid cocci fermented mannitol and a like proportion salicin. Of the udder type 76 per cent fermented saccharose and 16 per cent produced acid in salicin; all failed to ferment mannitol. They found that the milk-souring organisms were able to multiply at a temperature as low as 10°C. The udder streptococci grew better at 38°C. but 82 per cent were able to grow at 43°C. Considerable stress is laid on the reducing properties of the lactic acid organisms. The lactic acid types were able to grow in milk containing methylene blue and other dyes and were able to reduce the substances. The udder streptococci failed to grow in the methylene blue medium. In milk containing litmus members of both groups reduced the substance, but the lactic acid streptococci reduced litmus before coagulation, the others after the medium had coagulated.

It will be noted from the foregoing brief résumé that the specific identity of the so called *Streptococcus lacticus* is more or less obscure. The work of Sherman and Albus did much to show that differences existed between the lactic acid and udder groups.

It was considered advisable to study the species or groups of species with the idea that there exists a clear-cut differentiation from the udder or mastitis streptococci. With this in view, milk from various sources was permitted to sour at room temperature and in the incubator. Plate cultures were made from the milk at various times. The isolated organisms were studied in the same type of medium employed in the study of mastitis and equine streptococci.

#### EXPERIMENTAL.

The following experiment may be regarded as a type. Bottled market milk was purchased and after mixing distributed into sterile wide mouth bottles. These were permitted to stand at room temperature. The milk was examined at various intervals; usually an unopened bottle was chosen each time. A sample of the fresh milk was plated at once; subcultures were made from colonies resembling streptococci. In Table I the protocols of five samples of milk are given.

TABLE I.

Sample.	Length of time left at room temperature.	Acidity of whole milk.		Acidity of whey.		No. of organisms per cc.	Proportion of streptococci.	Subcultures referred to in Table II.
		per cent	per cent	Titration.	Hydrogen ion con- centration. <i>pH</i>			
A	days 0	1.3				2,160	5 per cent.	1, hemolytic. 3, " 4 A, non-hemolytic. 7, hemolytic. 13, " 15, non-hemolytic. 16, " 18, " 18 A, " 20, hemolytic. 21, non-hemolytic. 21 A, " 23, " 25, " 25 A, "
A	1	1.5				6,300,000	5 "	
A	2	7.5	6.0		4.7	220,000,000	90 "	
A	3	9.2	7.3		4.6	107,000,000	2 per cent hemolytic. 90 per cent.	
A	4	8.4	6.9		4.4	720,000,000	90 " 1 per cent hemolytic.	
A	7	9.0	7.2		4.7	760,000,000	90 per cent.	
A	14	8.1	7.4		4.8	960,000,000	85 " "	
B	0					7,500	5 per cent hemolytic.	27, hemolytic. 28, "
B	1	5.3	4.2		5.3	12,800,000	90 per cent.	
B	2	9.1	6.8		4.8	3,500,000,000	1 per cent hemolytic. 95 per cent. Few hemolytic colonies.	29 A, non-hemolytic. 30, " 30 A, "

B	3	9.6	7.7	4.6	5,000,000,000	90 per cent non-hemolytic.	31, non-hemolytic. 32 A, "
B	5	7.5	7.0	4.9	3,000,000,000	90 " " Less than 1 per cent hemolytic.	33, " 34, hemolytic.
B	7	9.0	8.6	4.8	1,500,000,000	95 per cent non-hemolytic. 1 " " hemolytic.	35, non-hemolytic 36, hemolytic.
C	2					95 " " non-hemolytic.	26, non-hemolytic. 26 A, "
D	2 (incubator).	8.0	7.9	4.7	3,000,000,000	90 " "	45, "
E	2 (room).	8.2	7.6	4.9	500,000,000	50 " "	43, "

It will be noted from the table that Sample A was milk of high quality. It soured slowly, but between the 24th and 48th hours there appeared in the plates enormous numbers of non-hemolytic streptococci. These organisms practically overwhelmed all others and persisted throughout the observation. The hemolytic (mastitis) streptococci which were in the unsoured sample persisted until the 4th day. The non-hemolytic udder type was still found after 3 days of souring. Perhaps both types survived throughout, but could not be detected on account of the great number of other organisms. Sample B reveals about the same condition. The coagulation was more rapid and due to the growth of enormous numbers of non-hemolytic streptococci. The hemolytic streptococci observed in the fresh sample persisted for the 7 days allotted to the observation. They were unable to multiply with the same facility as the others. In Samples C, D, and E the non-hemolytic organisms also predominated.

It soon became apparent that souring could not be attributed to the common type of streptococci met with in udder and bottled milk. The udder types were able to persist through at least part of the souring period but not in sufficient numbers to be the cause of the phenomenon.

The detailed fermentative characters of the various strains noted in the protocols are given in Table II. It must be recognized that these cultures are merely types. Others have been studied but fail to show further differences from those recorded.

The figures given in Tables II and III represent the total acidity reached in the various tubes. All the fermented bouillon was of the same lot. Duplicate series were inoculated and incubated at 38°C. and at room temperature. The recorded figures represent the maximum acid production at the temperature best adapted for growth. Thus, Cultures 1, 3, 4 A, 7, 13, 18 A, and 20 grew better and produced more acid at 38°C., therefore the acid produced by them at this temperature has been recorded. On the other hand, all the other organisms adapted themselves better to temperatures of 20–22°C., so that the acid production has been given after incubation at this temperature.



TABLE II.  
*Fermentative Characters of Streptococci Noted in Table I.*

Culture No.	Production of acid in.								Hemolysis.
	Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannitol.	Salicin.	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
1	5.6	4.7	4.7	5.2	1.0	1.0	1.0	1.0	+
3	3.8	3.3	2.6	3.6	1.1	1.0	1.1	1.0	+
4 A	5.1	4.1	5.6	4.8	1.1	1.0	1.1	3.6	—
7	5.0	4.2	4.7	4.6	1.0	1.0	1.0	1.0	+
13	5.6	4.9	4.6	5.2	1.1	1.1	1.1	1.1	+
18 A	4.9	4.4	4.5	4.4	1.1	1.1	1.1	2.3	—
20	4.6	4.4	4.7	3.6	1.0	1.1	1.1	1.0	+
18	6.9	6.0	1.1	6.9	1.1	1.0	4.6	4.4	—
21	6.7	3.8	1.1	5.8	1.0	1.0	5.8	5.8	—
25 A	6.7	5.4	1.1	6.1	1.1	1.1	4.8	4.6	—
26	6.6	5.8	1.1	5.2	1.1	1.0	4.2	4.0	—
26 A	6.8	5.4	1.1	6.6	1.1	1.1	5.9	4.5	—
30	6.9	5.9	1.1	6.5	1.1	1.1	4.8	5.0	—
31	6.7	5.7	1.1	5.9	1.1	1.1	3.5	3.8	—
32 A	6.6	5.8	1.1	6.1	1.0	1.1	6.5	6.5	—
33	6.7	6.1	1.1	5.6	1.1	1.1	4.1	5.0	—
35	6.8	6.2	1.1	5.8	1.1	1.0	3.6	5.3	—
43	6.5	6.1	1.1	6.0	1.1	1.1	4.4	5.0	—
45	6.8	6.1	1.1	5.9	1.1	1.1	4.9	5.0	—
15	6.5	6.1	6.4	5.4	1.0	1.1	4.8	4.8	—
16	7.1	5.7	5.3	4.7	1.1	1.1	3.9	4.6	—
23	6.8	6.2	6.4	5.6	1.0	1.1	4.3	5.8	—
29 A	6.1	5.0	5.4	4.0	1.1	1.1	3.4	5.0	—
21 A	6.5	4.8	6.1	6.5	1.1	1.1	1.2	6.5	—
25	7.0	6.4	6.6	5.8	1.0	1.1	1.1	5.8	—
30 A	6.0	4.4	4.9	5.2	1.0	1.1	1.1	5.4	—
27	6.6	5.0	1.1	5.1	1.0	1.1	1.0	5.4	+
28	6.7	5.2	1.1	5.3	1.1	1.0	1.0	5.4	+
34	7.0	5.2	1.1	5.2	1.0	1.0	1.0	6.0	+
36	6.5	5.0	1.1	5.0	1.1	1.0	1.1	5.4	+

It will be noted that Cultures 1, 7, 13, and 20 are hemolytic streptococci which were found in both fresh and sour milk. These strains are identical with those associated with mastitis. They are aggluti-

nated by their specific group serum. Culture 3 is similar to the low acid-producing streptococci found in the udder and in market milk. This species has been discussed in detail in a previous communication.<sup>12</sup> Cultures 4 A and 18 A are characteristic non-hemolytic mastitis streptococci which agglutinate with their specific antiserum. All these cultures promptly coagulate milk at 38°C. Litmus was not, as a rule, reduced even after coagulation. Where reduction occurred it was usually confined to the bottom of the tube. Since these well recognized udder and mastitis streptococci were unable to multiply to any great degree in soured milk, it is assumed that such is not their function. In other words, their true habitat is within the udder.

In contradistinction to the preceding group, a second still larger series, comprising Strains 18, 21, 25 A, 26, 26 A, 30, 31, 32 A, 33, 35, 43, and 45, made up the bulk of the flora of clotted milk. These organisms differed in morphology from the mastitis types. In bouillon the elements were usually elongated and arranged in pairs and short chains. Spherical forms were also observed. The mastitis cultures produced chains of cocci. The second group of organisms attacked dextrose, lactose, maltose, mannitol, and salicin, but failed to ferment saccharose, raffinose, and inulin. They produced more acid in dextrose than did the mastitis streptococci. Litmus was reduced before milk containing it was coagulated.

The next four strains (Nos. 15, 16, 23, and 29 A) were isolated from two samples only. They, too, differed from the mastitis streptococci morphologically. They produced large amounts of acid in media containing dextrose, lactose, saccharose, maltose, mannitol, and salicin. Litmus was reduced.

Cultures 21 A, 25, and 30 A also produced large amounts of acid. They attacked the same substances as the non-hemolytic mastitis streptococci but produced more acid. Litmus was reduced by these cultures. The characteristic rod-like and coccoid elements occurring in pairs and short chains are a further point in their differentiation. They grew best and produced a maximum acidity at room temperature. In addition they were not agglutinated by mastitis streptococcus serum.

<sup>12</sup> Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

The hemolytic streptococci (Nos. 27, 28, 34, and 36) which persisted throughout the souring of Sample B differed from the usual udder streptococci in several characters. Morphologically they

TABLE III.

*Comparative Acid Production by Streptococci in 1 Per Cent Fermented Broth at Incubator and Room Temperature.*

Culture No.	Incubator temperature.		Room temperature.	
	Titration.	Hydrogen ion concentration.	Titration.	Hydrogen ion concentration.
	<i>per cent</i>	<i>pH</i>	<i>per cent</i>	<i>pH</i>
1	5.6	4.8	5.0	4.9
4 A	5.3	4.9	4.6	4.9
7	5.0	4.7	5.1	4.9
13	5.8	4.8	4.8	4.8
18 A	4.9	4.7	4.4	4.9
20	5.6	4.7	4.8	4.9
18	6.0	4.7	6.9	4.6
21	5.8	4.8	6.3	4.4
25 A	5.3	4.7	6.7	4.5
26	5.0	4.8	6.6	4.6
26 A	5.4	4.7	6.8	4.6
30	5.9	4.8	6.9	4.6
31	5.2	4.8	6.7	4.6
32 A	5.1	4.9	6.6	4.6
33	5.4	5.0	6.7	4.5
35	5.6	4.9	7.0	4.5
43	6.0	4.6	6.5	4.5
45	5.7	4.8	7.0	4.4
15	5.8	4.8	6.9	4.6
16	7.0	4.6	7.1	4.5
23	5.6	4.9	6.8	4.5
29 A	4.4	4.9	6.1	4.5
21 A	6.0	4.7	6.5	4.5
25	5.9	4.7	7.0	4.5
30 A	4.4	4.8	6.0	4.6
27	7.0	4.5	6.9	4.5
28	7.0	4.7	6.7	4.5
34	7.1	4.9	6.9	4.5
36	7.1	4.7	7.0	4.4

resembled the sour milk types. They did not reduce litmus completely and failed to ferment saccharose. They grew as well at 38°C. as at room temperature.

It has been asserted that the sour milk organisms grew better and produced more acid at room temperature. Experimental evidence that this is the case is offered in Table III. Duplicate inoculations into fermented bouillon of the same lot containing 1 per cent dextrose were made; one set was incubated at 38°C., the other at room temperature. Titrations and hydrogen ion concentration readings were made after 7 days. All cultures grew well at both temperatures. The results are given in Table III.

The figures indicate that cultures of the mastitis type (Nos. 1, 4 A, 7, 13, 18 A, and 20) produced more acid when grown in the incubator. The reverse is true for the others except for the hemolytic strains (Nos. 27, 28, 34, and 36). That the true milk-souring streptococci grow better at ordinary temperatures (20–22°C.) certainly points to other sources of origin than the saliva, feces, or udder of cows. Cultures 27, 28, 34, and 36 differed from both groups in many characters. They may be saprophytic forms which have gained entrance into the udder and are still adapting themselves to a new environment.

The pathogenic properties of the sour milk streptococci have been studied by so many that further experimental evidence seemed unnecessary. Many have reported the entire lack of pathogenesis of the members of this group for laboratory animals. Unfortunately such findings cannot be regarded as a method of differentiation between mastitis and sour milk streptococci, since the mastitis streptococci are also lacking in virulence for species other than bovines.

#### DISCUSSION.

From the preceding observations it seems clearly established that the lactic acid group consists of at least three species of organisms. Each species possesses characters which differentiate it from the streptococci which are associated with mastitis and the low acid-producing udder forms.

Sufficient data to warrant the assertion that *Streptococcus lacticus* is an udder inhabitant have not been brought forth. Sherman and Albus first showed conclusively that there existed distinct group differences between the udder and the milk-souring streptococci. Aside from the specific cultural differences here reported a number of other points throw light on the question. The milk-souring types

have not been met with in milk drawn directly from the udder. Such organisms have not been found in the fresh bottled milk from a dairy where a great many examinations of udder milk have been made. Mastitis and udder streptococci are usually present in this bottled milk. The milk-souring group grows best and produces more acid at a temperature of 20–22°C. The udder streptococci prefer a higher temperature (38°C.).

Rogers and Dahlberg<sup>13</sup> found in milk in a few instances non-saccharose-fermenting streptococci which attack mannitol. They also studied over 50 strains of udder origin. Among these but one attacked mannitol without acidulating media containing saccharose. This organism liquefied gelatin.

Many have pointed to several possible sources of origin. The saliva, feces, and skin of cows have been mentioned particularly. The specific identifications were usually made on morphological findings. Many of the streptococci from the saliva, skin, and feces do resemble the lactic acid organisms in form, but they differ in other essentials. The writer has isolated 35 strains of streptococci from the saliva of cows. Although mannitol fermentation occurred in one-third of the cultures, every strain fermented saccharose. In addition the amount of titratable acid produced in dextrose rarely exceeded 4 per cent. The bovine fecal streptococci were characterized by their ability to produce large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Mannitol was not fermented. Streptococci from the skin produced acid in fermented broth containing raffinose, saccharose, and mannitol in addition to dextrose, lactose, maltose, and salicin. One feels justified in asserting that if *Streptococcus lacticus* inhabits these regions it exists in such small numbers that it cannot be detected. The souring of milk cannot, therefore, be attributed to the usual types found in the saliva, feces, or vagina or on the skin of cows.

Sufficient evidence exists to rule out the udder or mastitis group as the usual cause of souring under natural conditions. These organisms are readily detected in bottled milk, but cannot be found in plate cultures prepared from milk soured for 3 or 4 days. When.

<sup>13</sup> Rogers, L. A., and Dahlberg, A. O., *J. Agric. Research*, 1913–14, i, 491.

equal amounts of cultures of lactic acid and udder streptococci were added to tubes of sterile milk, the udder streptococci multiplied for 24 hours. Plates prepared from such milk after 48 hours at room or incubator temperature reveal only the lactic acid type. Under natural conditions probably other bacteria so alter the environment that the udder group is able to survive but not to multiply to any considerable degree.

I am inclined to agree with Kruse and others that the lactic acid organisms resemble more closely the genus *Streptococcus* than the genus *Bacterium*. Their growth on solid media and their fermentation characters resemble those usually associated with streptococci. The name *Streptococcus lacticus* I is suggested for the largest group (Table II), characterized by the fermentation of dextrose, lactose, maltose, salicin, and mannitol and their inability to attack saccharose, raffinose, and inulin. *Streptococcus lacticus* II may be considered as differing from the former group in its ability to ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin.

#### SUMMARY.

A well defined group of rod-like and coccoid organisms arranged in pairs and chains has been encountered in sour milk. The group comprises at least three species; the largest number ferment dextrose, lactose, maltose, mannitol, and salicin, and fail to ferment saccharose, raffinose, and inulin. A smaller number ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin. A few fail to attack mannitol. All three types grow luxuriantly at room temperature, coagulate milk, reduce litmus, and produce large amounts of acid in fermented bouillon containing dextrose.

Specific morphological and cultural differences exist between the lactic acid streptococci and those associated with mastitis and those occurring in the udder. The lactic acid organisms outgrow the udder streptococci in the milk-souring process. When both types are implanted in sterile milk the udder type soon disappears.

## PREPARATION OF COLLODION SACS FOR USE IN BACTERIOLOGY.

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(Received for publication, June 28, 1920.)

Metchnikoff, Roux, and Taurelli-Salimbeni<sup>1</sup> state that Fick in 1856 was the first to suggest the use of semipermeable collodion membranes in biology. Certainly a general interest in the properties of these membranes from the bacteriological standpoint dates from their own report in 1896 of the intoxication of guinea pigs from the diffusion of cholera toxin through the walls of collodion sacs containing cultures of the vibrio and placed in the peritoneal cavity. Since then a considerable number of articles on collodion sacs have appeared, which it is not within the province of this paper to review. It suffices here to note that the use of collodion sacs has been almost constantly attended with difficulties in manufacture, standardization, and mode of employment which have precluded their widespread use in bacteriology. There is at present no established bacteriological technique involving the intraperitoneal implantation of collodion sacs.

Heretofore most of the studies, whatever their primary object, have come at last to deal mainly with the properties of collodion sacs themselves, especially their permeability to a variety of substances, rather than with their use as an instrument for the furtherance of bacteriological research. The exceptions, however, are noteworthy. In addition to the demonstration of a soluble diffusible toxin of the cholera vibrio, noted above, mention may be made of Vincent's<sup>2</sup> enhancement of the virulence of saprophytes, *B. megaterium* and *B. mesentericus*, by collodion sac passage through rabbits; of Nocard and Roux's<sup>3</sup> demonstration of changes in collodion sac contents suggestive of the multiplication of inoculated material from pleuropneumonia of cattle; and of Nocard's<sup>4</sup> studies on the relation of human to avian tuberculosis by the growth of the human bacilli in sacs in the peritoneum of the chicken. All these papers appeared in 1896 or 1898 from the Pasteur Institute and with a few exceptions represent the total of the contributions made to bacteriology by the intraperitoneal use of collodion sacs.

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<sup>1</sup> Metchnikoff, E., Roux, E., and Taurelli-Salimbeni, *Ann. Inst. Pasteur*, 1896, x, 257.

<sup>2</sup> Vincent, M. H., *Ann. Inst. Pasteur*, 1898, xii, 785.

<sup>3</sup> Nocard, M., and Roux, E., *Ann. Inst. Pasteur*, 1898, xii, 240.

<sup>4</sup> Nocard, M., *Ann. Inst. Pasteur*, 1898, xii, 561.

Collodion sacs seem to afford a general method for the cultivation of bacteria in pure culture under conditions approaching the invasion of a host; the bacteria are free to yield soluble diffusible products which might affect the animal and to obtain nutritive substances from the animal tissues, yet they are protected against phagocytic attack and, to some extent certainly, from exposure to antibodies. The successful transmission of one human infection after another to animals previously supposed to be immune emphasizes the importance of new methods and new channels of infection, and, in view of the experiments from the Pasteur Institute already mentioned, suggests the further use of collodion sacs for the maintenance of bacterial growth intraperitoneally.

It was with these considerations in mind, and especially with the object of developing another method by which the inciting agent in certain diseases of unknown origin might be sought, that we have tried to obtain a standardized technique for the manufacture and manipulation of collodion sacs. The requirements are that sacs of suitable size and shape may be easily made in large numbers, that they shall be uniformly strong and highly permeable, and that they may be easily sterilized and handled without danger of contamination before and after incubation in the animal body. In the method to be described these problems have been given especial consideration. While methods formerly in use have been freely drawn upon, certain essential modifications have been found necessary, and it appears desirable to report the technique in detail so that it may be followed with exactness.

Following the method of Prudden and McCrae,<sup>5</sup> as modified by Harris,<sup>6</sup> the collodion sacs here described were made on a gelatin capsule foundation which was then dissolved out with hot water. The procedure is as follows:

#### *Preparation of the Sacs.*

A small piece of thin walled glass tubing 5 by 0.5 cm., the edges of which have been smoothed in the flame, is heated slightly at one end and pressed against the rounded end of the cap of a large veterinary

<sup>5</sup> McCrae, J., *J. Exp. Med.*, 1900-01, v, 635.

<sup>6</sup> Harris, N. MacL., *Centr. Bakt., It. Abt., Orig.*, 1902, xxxii, 74.



capsule (4.8 by 1.6 cm.),<sup>7</sup> to which it adheres. With a hot wire a hole is burned through the cap into the glass neck. The open end of the body of the capsule is momentarily dipped just beneath the surface of very hot water, applied to the cap, and thus sealed in with an overlap of about 2 mm. Slight suction with the lips discloses whether the joint is tight. If not, a drop of hot water is touched to the leak. After drying, this framework is ready for the first dip in collodion.

Collodion solutions of two different consistencies are required, one for reinforcement, the other for the permeable membrane. After some experiment Squibb's collodion U. S. P. IX was found satisfactory, though a 4 per cent collodion, as a basis, may be made up from the formula, absolute alcohol 25 parts, ether 75 parts, pyroxylin 4 parts. Squibb's 4 per cent collodion or this solution of pyroxylin is too thin to give a proper membrane with one dip, but higher percentages of pyroxylin do not readily dissolve, and it is found most convenient to obtain the thicker solutions by evaporation. This is accomplished under vacuum with the application of gentle heat (water bath). The collodion boils rapidly, and evaporation is measured by the loss in volume.

The 12 per cent collodion required for the permeable membrane and the 14 to 15 per cent collodion required for reinforcement are obtained by evaporating 4 per cent collodion to one-third or less of its original volume. For evaporating and for dipping, museum jars 20 by 6.5 cm.,<sup>8</sup> with clamped tops and rubber gaskets, are used.

Of the entire surface of the gelatin capsule, the body is to be covered by the permeable membrane of the sac, and the cap by the heavier impermeable wall of collodion which serves to strengthen and support the membrane. This impermeable part is made first by dipping the inverted gelatin capsule (held by wedging the somewhat conical body into the end of a test-tube) neck downward into the 15 per cent collodion to a depth that just covers the joint between body and cap.<sup>9</sup> The capsule is dipped and withdrawn slowly to avoid air bubbles in

<sup>7</sup> Parke, Davis and Company, Detroit, Mich., Empty Capsules No. 12. A capsule of any suitable size may be used.

<sup>8</sup> Whitall Tatum and Company, Philadelphia, Pa.

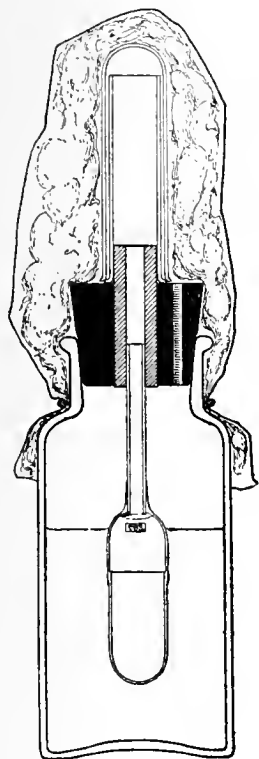
<sup>9</sup> The open end of the glass neck may be covered with a bit of adhesive plaster.

the angle between neck and shoulder and to allow excess collodion to run down the neck. After a few moments the collodion sets and ceases to flow, and the capsule, still held in the test-tube mouth, is inverted and set aside to dry. Before drying is complete the excess collodion is cut away from the glass neck with a knife, leaving a collar 7 mm. high that effectually joins sac and neck together when it dries. This heavy, dry collodion membrane seals and reinforces the joints between body and cap and between cap and glass neck.

The sacs may be identified by means of small numbered paper labels sealed into the wall. Tiny rectangles of very thin paper are numbered with India ink. These are cut apart, curved slightly on a blotter with the rounded end of a test-tube, dipped with forceps into thin collodion and applied to the dry membrane just below the shoulder, where they adhere and become a permanent part of the wall (Text-fig. 1).

The capsules are now ready for the final dip. They are to be handled, after the sacs are made, in 120 cc. (4 ounce) wide mouth bottles with rubber stoppers. These stoppers have one hole centrally placed for the glass neck, and a second peripherally, which equalizes pressure in the bottle and the sac. On account of its greater elasticity and resistance to autoclaving, a length of stethoscope tubing makes a good core for the central hole in the rubber stopper. The hole is cut with a cork borer slightly smaller than the tubing, which is seized with forceps, pulled through with a projection of 1 cm. on the upper side of the stopper, and cut off flush with the bottom. The use of the projection will be described later.

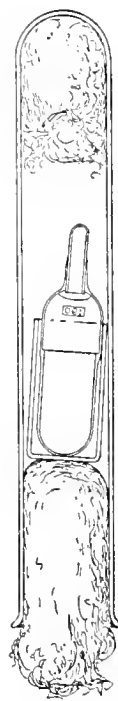
The neck of the reinforced capsule is inserted into the rubber stopper. The capsule is then slowly lowered into 12 per cent collodion in the jar with a rotary to and fro motion which reduces the incidence of air bubbles. It is immersed to the neck in the collodion, so that the joints at neck and body are reinforced. On withdrawal, the coated capsule is suspended above the collodion by means of a slotted card slipped under the stopper and is allowed to drain a definite length of time, as will be described. The membrane dries very little during this draining because of the ether-alcohol tension above the collodion. If a drop hangs from the capsule at the end of the drainage time it is removed by touching it to the surface of the collodion solution. The



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 1. Cross-section of a completed collodion sac in its protecting bottle. The various steps in the preparation of the sac are explained in the text.

TEXT-FIG. 2. A Pasteur pipette prepared for dry sterilization.

TEXT-FIG. 3. A sac in its cup and test-tube, after the neck has been sealed off in a small blast flame.

capsule is rapidly withdrawn from the jar and inverted to dry in the air for 1 to 2 minutes. It is then lowered into 95 per cent alcohol in a 4 ounce wide mouth bottle, where the collodion membrane finally sets.

The capsule with its collodion coating may remain in the alcohol indefinitely, but a few minutes are sufficient to extract the remaining ether and saturate the membrane with 95 per cent alcohol. As the viscosity of the collodion and the drainage determine the thickness of the sac, so the drying and the alcohol treatment determine its permeability.

After treatment in 95 per cent alcohol the capsule is plunged into cold water until the alcohol is removed and the gelatin capsule framework softens, a matter of an hour or less. The gelatin is then thoroughly washed out with a stream of hot water. For this purpose we have used a board with holes to accommodate twelve stoppers, resting on a basin of hot water. From the faucet, distributing tubes, ending in glass capillaries which are just long enough to reach into the sacs but not to pierce their bottoms, carry in the hot water and so wash the dissolved gelatin out through the neck. An hour or so of washing leaves the completed sac, attached to its glass neck by the collar of heavy collodion. It is then emptied with a Pasteur pipette and tested for strength and imperfections. Each sac must withstand an internal pressure of at least 25 cm. of mercury (5 pounds per square inch). The empty sac is immersed in distilled water in a 4 ounce bottle, and the second hole in the stopper is connected by a stub of glass tubing with a vacuum pump and gauge. The air in the bottle is exhausted 25 cm. A stream of air bubbles from the surface of the sac reveals any leak. If bubbles in the collodion were carefully avoided a leak is rare. Imperfect sacs are not worth repairing and are discarded.

The sac is then filled to the neck with distilled water and is ready for the autoclave. The expansion of water on autoclaving is sometimes sufficient to cause an overflow, and consequent loss of water on cooling. The projection of the stethoscope tubing above the stopper is therefore fitted with a short length of glass tubing open at the top. This forms an expansion chamber, from which the water drains back into the sac on cooling. To prevent contamination the glass tube is capped by a short, loosely fitting, inverted test-tube.

The top of the bottle is swathed in raw cotton and covered with paper, tied on. In this way contamination of either the inside or outside of the sac is avoided, and the sac is protected indefinitely for future use. Ten or twelve sacs are thus prepared at one time and then autoclaved at 15 pounds pressure for 30 minutes (Text-fig. 1).

### *Mode of Use.*

Before discussing permeability, which is the vital factor in the collodion sac technique, the mode of inoculation and peritoneal implantation may be described briefly.

The most convenient instrument for filling and emptying the sacs and preserving their contents is a Pasteur pipette. Those which we use are made in quantities in the laboratory from 24 cm. lengths of rather heavy glass tubing pulled out in the middle and sealed off to make two pipettes. A bulb about 2 cm. in diameter is then blown just above the shoulder. The requisites are very hot glass and gentle pressure. The tip is cut just to reach the bottom of a Noguchi culture tube (20 by 1.5 cm.), and a wrapping of cotton below the bulb acts as a stopper for the tube, in which the pipette is dry sterilized (Text-fig. 2). With these pipettes the sacs are filled, and after incubation the sac contents are similarly withdrawn and transferred to the test-tube which has protected the pipette from contamination. Then the pipette with its cotton collar serves as a plug for the tube and is used for subsequent withdrawal of fluid for examination.

Inoculation of small quantities of material into media in the sacs is accomplished with a platinum loop or a small sterile Wright pipette plugged with cotton. Such a pipette, flamed, and bent into a V saves the use of a Pasteur pipette in emptying out the distilled water from the sterilized sac preparatory to use. The bottle is inverted, the small arm of the pipette inserted upward through the neck, and gentle breath pressure expels the distilled water without danger of air contamination.

After being filled to the neck with a suitable medium and inoculated with bacteria or suspected material, the sac is withdrawn from the protecting bottle and the neck sealed off smoothly in a small blast flame. During this process and subsequently, contamination of the outside of the sac is avoided by carrying it in a short glass cup with

a flat bottom so that it will stand upright. Those used in the present work were made from 6 dram homeopathic vials cut off with a hot wire and a plunge into cold water. They are wider than the sacs and about 3 cm. long, so that the shoulder and neck project. The cups are sterilized by dry heat in wide test-tubes. When the neck of the sac is to be sealed off, one hand holds a cup containing the sac, while the other hand manipulates the rubber stopper. The cup, with its sac, is reinserted into the inverted test-tube until the neck has cooled (Text-fig. 3). The neck is then painted with a layer of collodion, which fuses with the collodion collar and so prevents the possibility of leakage between collar and neck. After a few minutes drying the sac is ready for intraperitoneal insertion. This is done aseptically under full ether anesthesia through a short incision in the abdominal wall above the umbilicus. The surgical technique need not be described, except to say that the incision should be sewed up firmly in layers. Metal skin clips save time. A dressing of cotton and collodion is sufficient. No bandage is required.

As many as eight such sacs have been tolerated by an adult rabbit without any apparent discomfort. The tissue reactions which often occur around the sacs will be discussed later. After an appropriate incubation period the rabbit is killed by an occipital blow and the sacs are recovered through a wide abdominal incision. They are again dropped into the glass cups, opened at the shoulder with a hot wire loop, and their contents transferred to a Noguchi tube by means of its Pasteur pipette.

#### *General Characteristics of the Sacs.*

Sacs made as just described have a capacity before autoclaving of from 6.5 to 7 cc. The upper third is a tough, impermeable wall which supports the glass neck and the permeable bottom. The lower two-thirds, with a surface area of about 14 sq. cm., is a thinner, moist, elastic membrane of high permeability. It is heavy enough, however, to stand immersion in water without collapsing and to withstand an internal pressure of 5 pounds per square inch. Such sacs rarely break during incubation. The walls of the sac are colorless and transparent.

Autoclaving at 15 pounds pressure for 30 minutes causes a uniform shrinkage to a capacity of 4.7 to 4.2 cc., corresponding to a diminution in surface area of approximately 25 to 30 per cent. Permeability is somewhat diminished but remains high. The shape and transparency of the sac are unaltered. The sacs may be kept sterile in distilled water in their bottles for months without appreciable loss in permeability, but the walls tend to become more brittle and inelastic.

### *Permeability.*

The permeability of the membranes has been tested in a number of ways. The quantitative test by which variations in materials and methods of manufacture were compared consisted in dialyzing a 2 M solution of sodium chloride within the sac against 20 or 25 volumes of distilled water and titrating a sample of the dialysate from time to time with 0.02 M silver nitrate solution, sodium chromate being used as indicator. The quantitative relations were such that when equilibrium was reached 1 cc. of the dialysate would precipitate 5 cc. (or 4 cc.) of the silver solution as silver chloride.

In 1915 Brown<sup>10</sup> contributed a careful study of collodion membranes and showed in particular that the permeability of membranes could be accurately controlled by complete drying, followed by immersion in ethyl alcohol of a definite dilution. Alcohol dilutions below 30 per cent confer practically no permeability on an air-dried membrane. 95 per cent alcohol produces a high degree of permeability. In preliminary experiments Brown's results were fully confirmed, and the value of his discovery was appreciated. His membranes, however, were prepared for chemical dialysis and did not require sterilization. On trial it was found that air-dried membranes, rendered permeable by alcohol treatment, lost their permeability again upon sterilization by heat. On the other hand, sacs which were not allowed to dry before immersion in alcohol lost little in permeability by sterilization in the Arnold sterilizer or the autoclave. Since highly permeable sacs were desired, 95 per cent alcohol was used as a routine.

*Viscosity of the Collodion.*—The percentage of pyroxylin in the collodion determines its viscosity, and viscosity in turn determines

<sup>10</sup> Brown, W., *Biochem. J.*, 1915, ix, 591.

the thickness of the collodion coating that will adhere to the dipped capsule. On account of rapid evaporation of the solvents the solution must be tested frequently and maintained at approximately the proper density. Commercial collodions vary considerably in density, and it is not a safe rule to evaporate a so called 4 per cent or U. S. P. collodion to one-third of its volume to obtain a 12 per cent solution.

The percentage of pyroxylin in the solution may be measured directly by weighing a specimen before and after evaporation to dryness. But this is not a convenient method, except as a check and to standardize a simple viscosimeter by which the thick collodions may be readily tested and compared.

The viscosimeter which we use consists merely of a 15 cm. length of Pyrex glass tubing, with square-cut ends, the internal diameter of which happens to be 0.365 cm. Near the middle of this tube file marks measure a distance of 5 cm. The tube is dipped into the collodion to be tested, a column is drawn up by suction well beyond the upper file mark, and the tube is withdrawn. As the column is then released in the vertical tube a stop-watch is used to time the meniscus over the measured distance of 5 cm. 12 per cent collodion at 20°C. requires  $15 \pm$  seconds to flow past the marks on the viscosimeter used.

Having standardized such a simple instrument it is easy to maintain thick collodion at the proper density by frequent tests and the addition of ether and absolute alcohol 3:1 to compensate for evaporation.

An even simpler method of determining viscosity, and one which is probably sufficiently accurate for the purpose, is to time the flow of collodion from a dipped capsule until the stream breaks and is succeeded by a series of drops. This time interval increases rapidly with the density of the collodion. In one test, for example, it increased from 12 seconds with a 10 per cent collodion to 1 minute, 15 seconds with the same collodion evaporated to approximately 12.7 per cent. The stream should flow about 1 minute before breaking.

The heavier collodion used for the neck and shoulder of the sac need not be standardized. We use it so thick that it will just flow smoothly, with no tendency to "jell." Collodion that has been used for permeable membranes until it has lost its clear transparency will serve the purpose.

*Determination of the Draining Time.*—The length of time that drainage is allowed to proceed influences the thickness of the collodion



coating and the evenness of its distribution over the surface of the capsule. Provided the first few drops are permitted to fall after the stream of collodion has broken, little is gained by prolonging the drainage. As will be shown later, slight variations in thickness are of minor importance, and sufficient uniformity is obtained by allowing a definite number of drops to form and fall.

*Experiment 1.*—From five sacs, after dipping, an unbroken stream of collodion flowed for an average of 1 minute, 16 seconds. Successive drops then fell after 1 minute, 26 seconds; 1 minute, 52 seconds; 2 minutes, 32 seconds; and 3 minutes, 29 seconds on the average. After 2 drops had fallen, 40 seconds were required to reduce the thickness of the membrane by the amount of 1 drop of collodion, and 57 seconds were required for the following drop to form.

It is hardly profitable to prolong the drainage time beyond the separation of the first few drops.

*Effect of the Drying Time on Permeability.*—

*Experiment 2.*—Five collodion sacs were made on No. 12 gelatin capsules by dipping once in 12 per cent collodion, draining 2 minutes, and drying for various intervals, as shown in Table I, before immersion in 95 per cent alcohol for 1 hour.

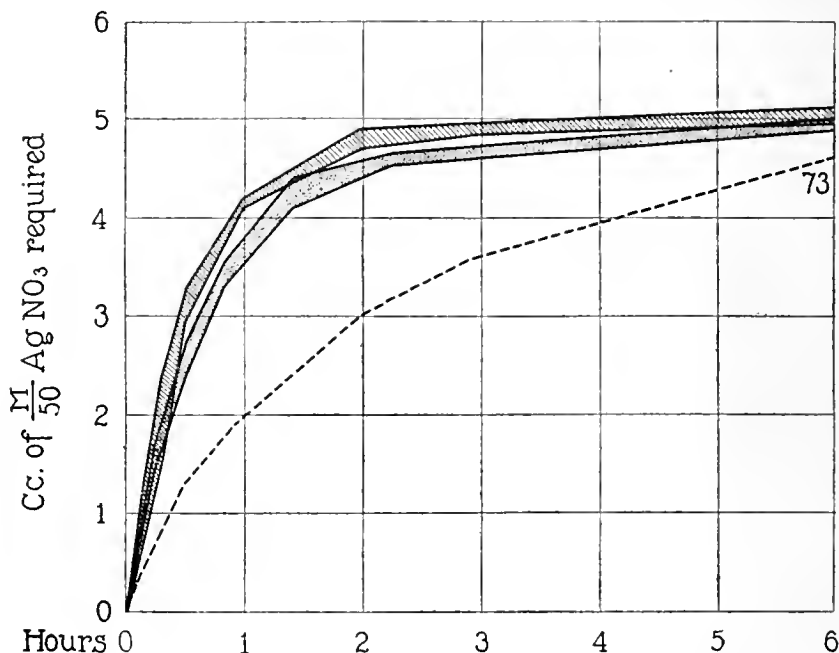
TABLE I.

Sac No.	Drying time.	Sterilization.	Capacity.	
			Before sterilization.	After sterilization.
81	0	Arnold sterilizer, 3 hrs.	cc. 6.4	cc. 4.6
76	15 sec.	Autoclave, 30 min.	6.2	4.8
82	30 "	Arnold sterilizer, 3 hrs.	6.4	4.7
80	1 min.	Autoclave, 30 min.	6.1	4.6
73	1 hr.*	Arnold sterilizer, 3 hrs.	7.3	6.2

\* Completely dry in 1 hour.

The permeability of the sacs to sodium chloride was determined both before and after sterilization and is shown in Text-fig. 4. Sodium chloride diffused much more rapidly through the sacs which were not completely air-dried before the alcohol treatment. Sterilization, whether in the Arnold sterilizer at 100°C. or in the autoclave at 15 pounds pressure, did not greatly impair the permeability, notwithstanding the shrinkage which accompanied the process. The air-dried sac,

No. 73, on the other hand, shrank less during sterilization, but its permeability was so far lost in the Arnold sterilizer that no measurable amount of sodium chloride had passed through it in 6 hours, and only 1.30 units (on a scale of 5) had passed in 48 hours.

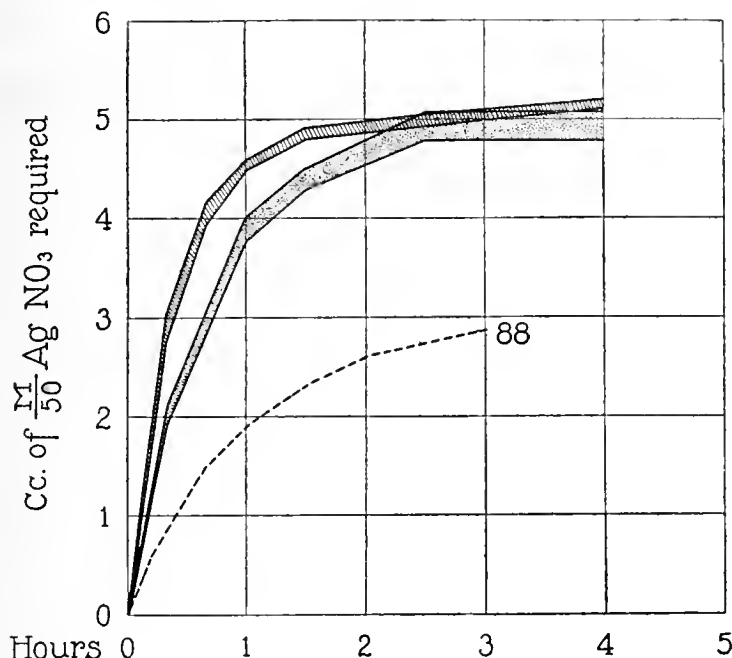


TEXT-FIG. 4. Experiment 2. The cross-hatched area covers variations in permeability of Sacs 81, 76, 82, and 80 (Table I) before sterilization. The stippled area covers variations in permeability of these sacs after sterilization. The broken line is the permeability curve of Sac 73, dried before the alcohol treatment. Sac 73, after sterilization, passed no measurable amount of sodium chloride in 6 hours.

*Experiment 3.*—In a similar experiment four sacs were dipped once, drained 3 minutes, dried 1, 2, 3, and 4 minutes respectively, and treated with 95 per cent alcohol over night. Their capacities before sterilization were 6.3, 6.1, 6.0, and 6.5 cc., and they shrank to 4.9, 4.7, 4.7, and 5.3 cc. in the autoclave. Their permeability before and after autoclaving is shown in Text-fig. 5.

From these two experiments it is evident that variations in the drying time have little influence on permeability, provided the membrane is still moist when immersed in alcohol. Sacs dried for 30

seconds to 1 minute are the most satisfactory. If plunged immediately into alcohol after drainage they tend to wrinkle and to show a smoky bluish opacity, and they are not so tough and strong as the partly air-dried sacs.



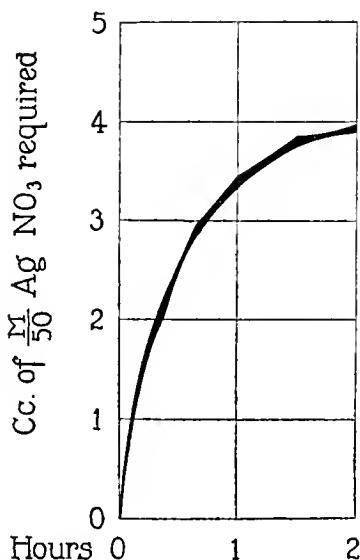
TEXT-FIG. 5. Experiment 3. The cross-hatched area covers variations in permeability of four sacs before sterilization. The stippled area covers variations in permeability of the same sacs after sterilization. The broken line is the permeability curve of Sac 88, not treated with alcohol before sterilization.

*Importance of the Alcohol Treatment.*—Other experiments indicate that treatment in 95 per cent alcohol for as short a time as 1 minute is sufficient to insure sacs of high permeability. It is only necessary that the alcohol should replace the ether-alcohol solvent of the colloid before the membrane is set by immersion in water. The importance of the alcohol treatment is illustrated in Text-fig. 5 by the permeability curve of Sac 88, which was drained 1.5 minutes, dried 30 seconds, and then plunged into water without the preliminary

immersion in alcohol. Although this sac was made of the same collodion as the others and shrank only from 6.9 cc. to 6.5 cc. during sterilization, its permeability to sodium chloride was much less than that of alcohol-treated sacs.

*Shrinkage of the Sacs during Sterilization.*—The hot water used to wash out the gelatin framework of the sacs causes an initial loss in capacity from 7+ cc. to about 6.6 cc. Sterilization by heat further shrinks the membrane to an average capacity of 4.5 cc. The loss in capacity and permeability due to heating is absolute and does not continue once the limit is reached.

*Experiment 4. Effect of Re-Autoclaving.*—Four sacs, dipped once in 12 per cent collodion, drained 1.5 minutes, dried 30 seconds, and immersed in 95 per cent alcohol for 1 hour, were autoclaved at 15 pounds pressure for 30 minutes. Two of the sacs were then re-autoclaved under similar conditions. The capacity of the first two sacs was 4.5 and 5.1 cc. respectively, of the second two 4.4 and 5.05 cc. All four sacs had a practically identical permeability as shown by Text-fig. 6. The curve incidentally illustrates the uniformity of collodion sacs made as described above.



TEXT-FIG. 6. Experiment 4. The solid black area covers variations in permeability of four sacs, two of which were autoclaved once, the other two autoclaved twice.

The relation between the diminution in capacity and in surface area of the sacs on sterilization is shown in the following experiment.

*Experiment 5.*—Eight sacs made in an identical manner from the same collo-dion solution were divided into two groups of four each. After volume measurements had been taken, four of the sacs were autoclaved and their capacities again determined. All the sacs were then cut open so that they could be flattened out, shadowgraphs were made on photographic paper, and the areas measured with a planimeter. The average measurements are shown in Table II, in which are included similar figures for two sacs that were completely dried before the alcohol treatment.

TABLE II.

Condition and No. of sacs.	Volume.				Surface.			
	Before autoclaving.		After autoclaving.		Before autoclaving.		After autoclaving.	
	Body.	Total.	Body.	Total.	Body.	Total.	Body.	Total.
	cc.	cc.	cc.	cc.	sq. cm.	sq. cm.	sq. cm.	sq. cm.
Two dried sacs.			4.9	6.95			14.3	21.9
Four undried sacs; not autoclaved.	4.5	6.72			14.0	21.6		
Four undried sacs; autoclaved.	4.45	6.67	2.72	4.5			9.6	15.8
Capacity loss.			39 per cent.	33 per cent.			31 per cent.	27 per cent.

It is seen from Table II that sterilization of the permeable sacs caused a loss of 33 per cent in capacity, corresponding to a surface shrinkage of 27 per cent. The losses were not proportionately distributed between the impermeable cap and the permeable body, which lost 39 per cent in capacity and 31 per cent in surface area.

This shrinkage on heating is due largely to the alcohol treatment of undried sacs. Dried sacs, even though treated subsequently with alcohol, shrink but little in the alcohol, as Brown<sup>10</sup> has shown, or in the autoclave (Table II). Undried sacs, not treated with alcohol, also shrink but little on sterilization. The part played by shrinkage in decreasing permeability has probably been overestimated. That the shrinkage itself is of minor importance is shown by a comparison

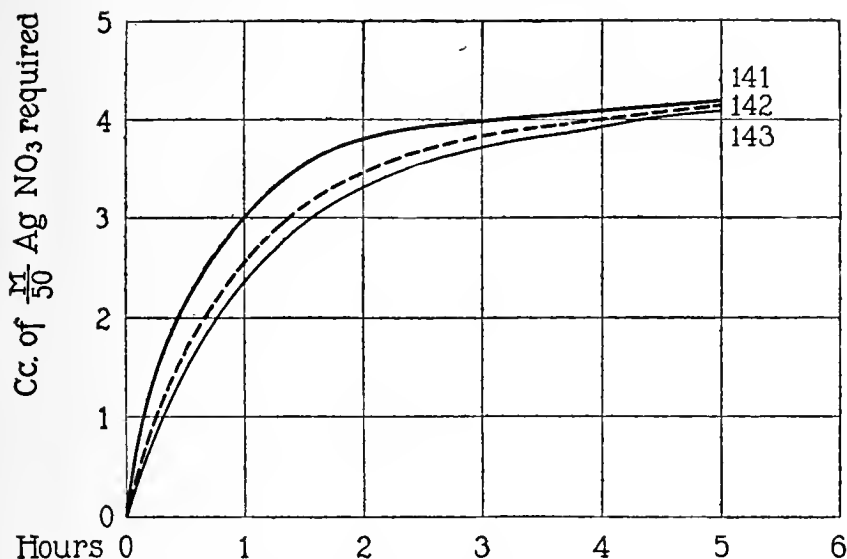
of the permeability of alcohol-treated undried sacs with that of sacs prepared by other methods.

In this connection it may be pointed out that titrations with sodium chloride do not give complete information with regard to the permeability of the sac walls. The sodium chloride molecules and their constituent atoms are of relatively small size, and the rate at which they diffuse through the permeable membranes suggests that the pores of the membrane must be several times the size of the molecules to permit such rapid passage. The volume shrinkage on sterilization is approximately 33 per cent, which corresponds to a diminution in permeable surface area of 31 per cent. Assuming that the pores diminish correspondingly, it is conceivable that while small molecules might still pass through with ease, other molecules which more nearly approached the unshrunk pores in size might now be entirely held back. So far, however, among the substances tested none has been found which passes through the unheated, but not through the heated membranes.

*Relation of Thickness to Permeability.*—Another factor emphasized in permeability experiments with collodion membranes is thickness. In the present method, within limits, this factor also is found to be secondary in importance to the alcohol treatment. The viscosity of the collodion solution and the drainage and drying time are the variables in determining thickness and have been experimentally controlled (Experiments 1, 2, and 3). A 12 per cent solution forms a sac of sufficient strength for intraperitoneal incubation. Thicker sacs, however, may be used without great loss in permeability.

*Experiment 6.*—Three collodion sacs were made on a gelatin capsule framework. No. 141 was dipped once in 12 per cent collodion, No. 142 twice, and No. 143 three times. The sacs were drained 2 minutes and dried 45 seconds between each dip. All were immersed in 95 per cent alcohol for 1 hour, washed, and autoclaved. Sac 141 was thin and transparent. Sac 142 was of medium thickness, with a slightly smoky opacity. Sac 143 was very thick and tough, with a smoky opacity. Capacities before autoclaving, 6.8, 6.1, and 6.4 cc. respectively; after autoclaving, 4.7, 4.0, and 4.2 cc. Such sacs in their thinnest area, just below the joint, were found to measure 0.09, 0.31, and 0.52 mm. in thickness after sterilization.

The results of permeability tests with 4 cc. of 2 M sodium chloride against 96 cc. of distilled water are shown in Text-fig. 7.



TEXT-FIG. 7. Experiment 6. The permeability curves, after sterilization, of three sacs of widely different thicknesses. Sac 141 dipped once in 12 per cent collodion, Sac 142 dipped twice, and Sac 143 dipped three times. In this experiment equilibrium in dialysis is represented by 4 cc. of silver nitrate. The error in the final readings is due to the removal of successive portions of dialysate for test. This loss in volume was not compensated during the experiment.

The difference between the sacs is less than would be expected from the comparative thickness of their walls and indicates that slight variations in thickness would be of no practical significance.

*Qualitative Tests of Permeability with Other Substances.*—In addition to the titrations of permeability with sodium chloride a number of qualitative tests with other substances have been made. These tests were incidental to other experiments and are therefore incomplete, but the results are useful in indicating the relative permeability of the sacs.

1. Various inorganic salts passed through the sacs with an ease approaching that of sodium chloride. With such substances the endosmotic pressure of a concentrated solution is so quickly lowered by passage through the sac that the level of liquid within the sac is hardly raised significantly before equilibrium is established.

2. Oxygen in solution rapidly diffuses through the sac wall and restores the color to methylene blue reduced by the action of dextrose broth. This fact probably explains the consistent failure of representative strict anaerobes to grow in permeable sacs intraperitoneally implanted. Haggard and Henderson<sup>11</sup> estimate the oxygen tension of the peritoneal cavity at about 45 mm. So far no method has been devised by which this handicap may be overcome.

3. During intraperitoneal incubation the hydrogen ion concentration of sac contents tends to come to an equilibrium with that of the peritoneal fluid. Ascitic fluid, dilute rabbit serum, and broth, for example, whatever their initial reaction, and  $\frac{M}{15}$  phosphate mixtures, in proportions to give various pH concentrations, have all been reduced to a pH of 7.4 to 7.5. *In vitro* it is found that the primary and secondary phosphates diffuse through the walls with equal facility. The dialysate comes almost immediately to the pH concentration of the phosphate mixture used.

4. Among the simpler organic compounds, dextrose alone has been tested. It soon shows its presence on the opposite side of the membrane, but diffuses more slowly than do inorganic salts and so gives opportunity for a considerable endosmosis before equilibrium is established.

5. Nutrient materials of meat infusion broth pass through the sacs with sufficient rapidity to promote a luxuriant growth of bacteria (*B. typhosus*, *B. pyocyaneus*) inoculated into distilled water. The straw-colored pigments of the broth are likewise diffusible. Nutrient materials are obtainable from body fluids in a similar manner. Heavy growths of *B. pyocyaneus*, *B. typhosus*, and Type I pneumococcus were obtained by incubating inoculated sacs of distilled water over night in a rabbit. *B. Pfeifferi*, lacking hemoglobin, did not grow under similar conditions.

6. The diffusible products of bacterial metabolism have not been determined by analysis. That the sacs are permeable to these products is shown by the tissue reactions which occur around actively growing cultures. The sacs themselves are practically inert, and it is common to find uninoculated control sacs lying free among the intestines without any observable irritation of the surrounding tissues. Sacs which contain living bacteria, on the other hand, are usually the center of an active proliferative process. They are found wrapped in folds of thickened and injected omentum or among intestinal adhesions, sections of which show fibrin deposits, accumulations of leucocytes, rarely to the extent of pus formation, localized hemorrhages, infiltration with fibroblasts, and newly formed capillaries; in short, the various elements and stages of a degenerative and regenerative tissue reaction.

7. No evidence has been observed that hemoglobin or other unsplit proteins will pass through the sacs. In two experiments in which highly potent antineurococcic serum was surrounded by a suspension of meningococci no agglutination occurred.

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<sup>11</sup> Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.



In summary, it may be said that the sacs as described are permeable to gases in solution, to inorganic salts, to dextrose, to certain protein-split products which are nutritive to bacteria, and to certain toxic products of bacterial metabolism, but they hold back antibodies, unsplit proteins, and formed elements such as bacteria and body cells.

#### SUMMARY.

A standardized method is described in detail by which collodion sacs suitable for intraperitoneal incubation and for other bacteriological experiments may be produced in large numbers, sterilized, and handled with convenience and the minimum danger of contamination. Various factors influencing permeability have been subjected to experiment. Like Brown, we found that immersion in alcohol is the most important factor, but the high permeability conferred by alcohol treatment is lost during heat sterilization if the membrane was previously allowed to dry. Quantitative experiments on the dialysis of sodium chloride, and simple tests with other substances indicate the general character of the membranes and their probable field of usefulness in bacteriology.



# A MECHANICAL MEASURING INSTRUMENT FOR STERILE LIQUIDS.

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*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, June 28, 1920.)

Part of the strain and tedium of immunological research is connected with the distribution of identical portions of a given liquid among a large number of tubes. Ordinarily this task is performed by means of a graduated pipette, in which the movement of the liquid is controlled by the ball of the finger and regulated by watching the meniscus as it passes the graduation marks. The process is time-consuming, and the precise control necessary to stop the meniscus exactly on the mark is a tax on eye and hand.

The instrument here described<sup>1</sup> was first devised as a substitute for the graduated pipette in immunological tests. Since it mechanically measures any amount of liquid within its capacity in a sterile manner it may find application also in serum and vaccine laboratories, in media preparation rooms, and wherever aliquot portions of a liquid are to be measured out.

As with the graduated pipette, the principle involved is the displacement of the required amount of liquid by a similar amount of air at atmospheric pressure. The air is measured by a graduated syringe, which may be set to deliver any amount within its capacity. Before coming in contact with the liquid, the air is twice filtered through raw cotton plugs. Provided the container is sterile all danger of contamination is eliminated.

## *Description of the Instrument.*

The syringe *F*, of any required capacity, is screwed into one outlet of a three-way stop-cock *H*, which also supports a rigid hollow sleeve

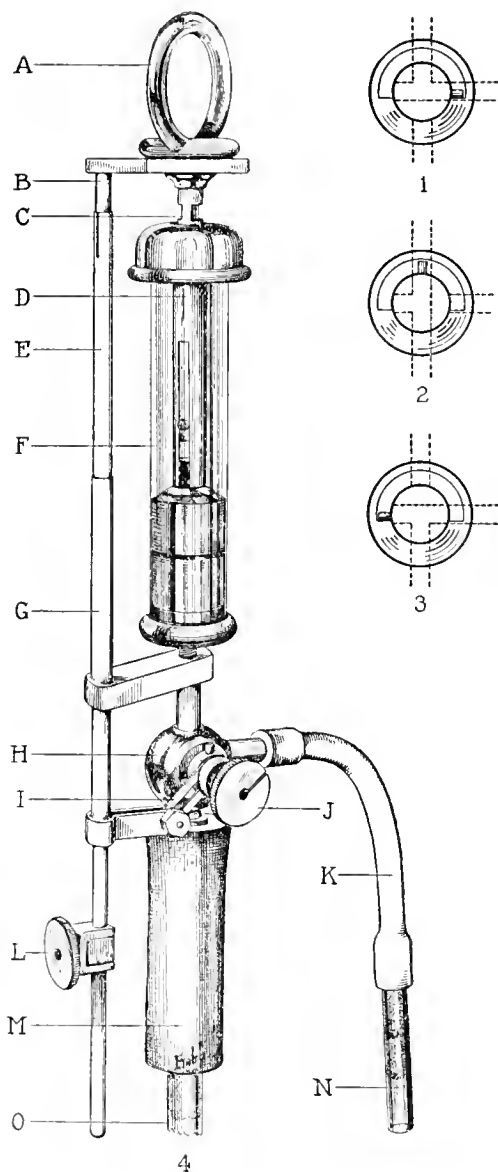
<sup>1</sup> The instrument may be obtained from the Central Scientific Company, 460 East Ohio Street, Chicago, Ill.

*G* and forms the frame of the instrument. This sleeve *G* guides an inner sliding sleeve *E* which operates the key of the stop-cock through the slotted arm *I* and is itself operated by friction contact with the sliding rod *B*. This rod engages the plunger rod *C* of the syringe, and by means of the adjustable lock-nut *L* limits its travel. The finger, slipped through the ring *A*, raises and lowers the plunger rod and operates the stop-cock mechanism. The plunger is surmounted by a slotted sleeve *D* in which the plunger rod fits loosely and operates the plunger only toward the end of the stroke in either direction. The object of these sliding mechanisms is to turn the key of the stop-cock before the plunger moves.

In Text-fig. 4 the instrument is pictured at the end of the downstroke. The stop-cock is open in the direction indicated in Text-fig. 2. A complete up- and downstroke may be followed in detail. As the finger, slipped through *A*, raises the plunger rod *C*, the rod *B* is raised also, carrying with it, by friction contact, the sleeve *E*. This sleeve in turn operates the key of the stop-cock through the slotted arm *I* so that during the travel of *E* the key turns from position 2 (Text-fig. 2) to position 1 (Text-fig. 1). During this first part of the upstroke the plunger does not move, since the plunger rod slides freely in the loose sleeve *D*. When the stop on *C* reaches the end of the slot in *D*, the sleeve *E* also reaches the frame *G*, and the key of the stop-cock reaches position 1. The stop-cock is now open for the admission of air to the syringe barrel through the side outlet of the cock and the short rubber tubing *K* and cotton filter *N*.

As the finger continues to raise the plunger rod the plunger is now raised also by the engagement of the stop on the rod in the end of the slot in *D*. The rod *B* slides through sleeve *E* until its travel is stopped by contact of the lock-nut *L* with sleeve *E*, which is already in contact with *G*. The plunger has now risen in the barrel a definite distance, determined by the position of the lock-nut *L* on the rod *B*, and a measured quantity of air at atmospheric pressure is contained within the syringe.

On the downstroke the first movement is again that of *A*, *B*, *E*, and *I*, operating to turn the key of the stop-cock back to position 2. This accomplished, the plunger rod *C* engages the sleeve *D* at the bottom of the slot and drives the plunger down. The air in the syringe



TEXT-FIGS. 1, 2, and 3. Various positions of the stop-cock key. (1) Position for the admission of air to the syringe; (2) for the expulsion of air into the liquid container; (3) for filling the container by suction.

TEXT-FIG. 4. The complete instrument. The lettering is explained in the text.

barrel is expelled into the container *O* attached to the heavy rubber tubing *M*, and displaces a corresponding quantity of liquid which is thus delivered by the apparatus.

The instrument may be set to deliver any volume of air, and consequently any volume of liquid, within the capacity of the syringe. To set it for a given volume the lock-nut *L* is loosened and the plunger raised exactly to the desired graduation mark on the syringe barrel. Then the lock-nut is set snugly against the sleeve *E*, in contact with *G*, and tightened.

Any sort of container with an inlet for the measured air and an outlet for the liquid may be used. The only requirement is that the system shall be air-tight except at the intake and outlet. The instrument may be attached to it directly or through a length of tubing. The elasticity or rigidity of the walls is of no consequence, since the system returns to atmospheric pressure after each delivery.

In immunological tests in which relatively small amounts of liquids are handled, a 5 or 10 cc. pipette may be used. The instrument is operated with one hand, and the pipette held and directed with the other. Larger amounts of liquid may be contained in flasks or bottles with a tightly fitting two-hole rubber stopper and a delivery tube reaching to the bottom. In such instances the instrument may be clamped in a frame and the rod *B* operated by a pedal, leaving both hands free.

When a pipette is used it is refilled as follows: The plunger is raised to the top of the stroke, and the split thumb-nut *J* partly unscrewed. It first releases the slotted arm *I*, and then, by friction, it turns the key of the stop-cock from position 1 to position 3 (Text-fig. 3). The stop-cock is now open from the side outlet to the pipette, which is filled by suction on *N*. The thumb-nut is again tightened, returning the key to position 1 and locking the arm *I* in place.

In order to minimize errors in measurement, attention should be given to several precautions in the use of the apparatus. (1) For exact measurements the syringe must be accurately calibrated and the lock-nut carefully set to bring the plunger exactly to the mark. (2) The system must be air-tight. Since the instrument itself need not be sterilized, the stop-cock and plunger may be lubricated with a heavy oil or grease. The addition of a little beeswax to the plunger

lubricant keeps the plunger in position at the top of the stroke until it is forced down by contact of the plunger rod. (3) An error is introduced by a difference in level between the surface of the liquid in the container and the point of outlet. Usually this error is negligible. A difference in level of 10 cm. causes an error of about 1 per cent. (4) Care must be taken that the air is not expelled too rapidly into the container so as to force the liquid out under pressure, lest more than the required amount be delivered by its own momentum. This is recognizable by the subsequent retreat of the liquid from the delivery tip, as equilibrium is reestablished. A complete stroke should be up, and then down, for each delivery. A small delivery tip lessens the error due to the adherence of the last drop of liquid. (5) When air and liquid are at different temperatures an error is introduced by expansion or contraction of the measured air on contact with the liquid. For accuracy, therefore, the air and liquid should be at the same temperature. Practically, when the instrument is handled with reasonable care, these sources of error are not encountered.

The facility with which sterile solutions may be measured out without contamination should be emphasized. The liquid does not pass through the instrument, and nothing comes in contact with it but twice filtered air.

#### SUMMARY.

An instrument is described by which aliquot portions of a liquid may be mechanically measured and delivered. It was devised especially for use in immunological experiments, to take the place of a graduated pipette in setting up serum tests. The instrument may be set for any quantity within its capacity and measures sterile liquids without danger of contamination. It may therefore find a wider application in other procedures requiring sterile measurements of small amounts of liquid.





# FACTORS INFLUENCING ANAEROBIOSIS, WITH SPECIAL REFERENCE TO THE USE OF FRESH TISSUE.

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## PLATE 2.

(Received for publication, June 24, 1920.)

Anaerobic cultivation of bacteria has in recent years developed into an important field of bacteriological research. Anaerobic methods have been extensively applied, for example, to the study of the bacterial flora of war wounds and to the investigation of diseases of unknown origin.

In these investigations the cultivation of anaerobes in tubes containing bits of fresh tissue has been extensively employed. Although study of some of the principles involved has led to improvements in method, a method for the quantitative examination of the factors involved is lacking and the establishment of practical rules and indications to meet the requirements of the more fastidious of this group of microorganisms is still to be attained. We shall attempt to show the influence of certain elements in promoting or in hindering the development of strict anaerobic conditions in culture tubes and so to indicate more exactly the value of these factors in anaerobic cultivation.

Aside from the use of mechanical methods for removing oxygen from ordinary media, much attention has been devoted in recent years to the cultivation of anaerobes in a fluid or semisolid medium containing a fragment of fresh sterile tissue, usually kidney, and overlaid with a layer of paraffin oil. The value of the tissue fragment was pointed out by Theobald Smith<sup>1</sup> in 1899, and rediscovered by Tarozzi<sup>2</sup> and Wrzosek<sup>3</sup> in 1905. The use of paraffin oil is credited to Legros.<sup>4</sup>

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<sup>1</sup> Smith, T., *J. Boston Soc. Med. Sc.*, 1898-99, iii, 340.

<sup>2</sup> Tarozzi, G., *Centr. Bakt., Its Abt., Orig.*, 1905, xxxviii, 619.

<sup>3</sup> Wrzosek, A., *Wien. klin. Woch.*, 1905, xviii, 1268.

<sup>4</sup> Legros, G., *Recherches bactériologiques sur les gangrènes gazeuses aiguës*, Paris, 1902; *Compt. rend. Soc. biol.*, 1902, liv, 1337.

In 1911 Noguchi,<sup>5</sup> using tall columns of serum water overlaid with paraffin oil in narrow tubes and containing a fragment of fresh tissue, was able to grow *Spirochaeta pallida* under strictly anaerobic conditions. He did not rely on the kidney tissue or the paraffin oil for the production of anaerobiosis, but for the first time employed with these a combination of hydrogen gas, vacuum, and pyrogalllic acid in an anaerobic jar.

Later, in the cultivation of the globoid bodies of poliomyelitis<sup>6</sup> it was found that the mechanical precautions could sometimes be omitted.

Following these reports the tissue-serum water or ascitic fluid tubes overlaid with paraffin oil came to be widely used as an anaerobic method and some investigators, seeking strictly anaerobic conditions, have disregarded the elaborate precautions which Noguchi employed. But the successful use of the tissue method alone, which appears to be simple, has been found to require considerable patience and experience, and the method has often suffered for lack of quantitative standardization and through misunderstanding of underlying principles.

A number of variables are involved. The first requisite for a study of these variables is a delicate and precise reversible indicator for the presence or absence of free oxygen in solution. The indicator should react in the presence of culture media, so that it may be added directly to the materials to be tested, and should not interfere with the reducing activity of other components of the medium or arrest the growth of test organisms. Methylene blue fulfills these requirements in a satisfactory manner.

Theobald Smith<sup>7</sup> in 1896 reported the reduction of methylene blue and other indicators in the closed area of fermentation tubes by sterile peptone broth. The presence of muscle or grape sugar and the application of heat increased the speed of the reaction. Peptone and dextrose water were inert. Spina<sup>8</sup> had already noted the reduction of methylene blue by nutrient gelatin, but not by agar.

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<sup>5</sup> Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

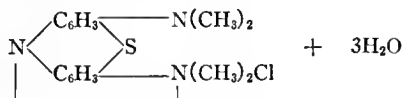
<sup>6</sup> Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

<sup>7</sup> Smith, T., *Centr. Bakt., 1te Abt.*, 1896, xix, 181.

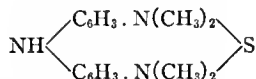
<sup>8</sup> Spina, A., *Centr. Bakt.*, 1887, ii, 71.

*Methylene Blue as Indicator.*

By the action of weak deoxidizing agents methylene blue (tetramethylthionine chloride)



is converted readily to colorless leucomethylene blue, or  $\alpha$ -(*p*)-tetramethyldiaminothiodiphenylamine



The reaction is reversible, and the conversions proceed rapidly at incubator temperature in the presence of free oxygen or under the attack of the deoxidizing agent after the free oxygen has been consumed.

- Experiments were made to determine the sensitiveness of the indicator and the conditions under which it reacts. Ordinarily 0.1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of 2 per cent dextrose peptone broth in a test-tube is placed in the anaerobic jar and the removal of free oxygen is indicated by the gradual decolorization of the dye. Since methylene blue is not decolorized under similar conditions in a medium of distilled water, we sought the ingredient in the dextrose broth which promotes the decolorization.

*Relation of the pH of the Medium to Its Reducing Activity.*—Beef infusion, 2 per cent peptone solution, and 2 per cent dextrose, with methylene blue, when boiled in a water bath to remove the air were not decolorized. Nor were mixtures of any two, or of all three of the ingredients. But so far in this experiment the hydrogen ion concentration of these mixtures had not been considered. The foregoing materials were retested in a solution of 0.01 M sodium hydroxide. Decolorization was immediate on heating to drive off the air, but the dye was resolved into simpler elements than the leuco form, thus destroying its value as an indicator.

2 per cent dextrose solution, colored with methylene blue, was mixed with an equal volume of buffer phosphate mixtures in  $\frac{M}{15}$  solution, to produce a range of pH concentrations from 6.6 to 7.8. Tubes of these mixtures were heated to expel the air and to increase the

velocity of reaction. The solutions of a pH of 7.4, 7.6, and 7.8 were quickly decolorized. On cooling and exposure to air the blue color as promptly returned. Similar but less rapid effects were obtained with the 2 per cent peptone solution. The beef infusion appeared to be inert.

The reducing action of dextrose in alkaline solution is well known as the basis of the Fehling and Benedict tests in urine analysis. The same reaction is utilized here. The routine use of methylene blue in dextrose peptone broth is simply a convenient method of assembling the materials in a weakly alkaline solution.

The delicacy of the reaction depends upon the feeble reducing power of the medium at the chosen hydrogen ion concentration and at incubator temperature. An equilibrium is established between the rate of diffusion of oxygen through the medium and the activity of the reducing agent. It is only when the access of oxygen is practically\* completely inhibited that the weakly alkaline dextrose solution is able to fix the remaining traces of the gas and then to attack the methylene blue.

*Comparison of Different Amounts of Methylene Blue.*—From 0.1 to 1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of dextrose broth was decolorized with practically equal rapidity in less than 24 hours in a McIntosh and Fildes jar.<sup>9</sup> For the purposes of this study, variations in the small quantity of the dye used in the medium were of little significance.

*Effect of Temperature.*—Like other chemical reactions, the rate of reduction is a function of the temperature. When access of free oxygen is prevented decolorization does not occur in 24 hours at 4°C. It proceeds slowly at room temperature (21°C.) and rapidly at 37°C. and higher temperatures.

*Estimation of the State of the Medium.*—It has been noted above that the state of the indicator, whether blue or colorless, depends upon the relative rate of the admission and diffusion of oxygen from the air and the activity of a reducing substance, e.g. dextrose, in the medium. The equilibrium between these two forces may be observed in a narrow test-tube by the depth below the surface at which the colorless zone begins. While methylene blue may not be decolorized in an oxygen-free liquid in the absence of a reducing substance, decolorization indicates a strictly anaerobic condition, and the return of the color is an index of the return of oxygen to the medium.

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<sup>9</sup> McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

With these indications of the sensitiveness of the reaction and of the factors that control its progress, we proceeded with a study of the various elements in the technique under investigation.

*Comparison of Liquid Paraffin Oil and Solid Vaseline as a Seal.*

The tissue medium technique as generally employed involves the use of liquid paraffin oil as a seal or supernatant to the medium. It was presumed that the oil, besides preventing evaporation, favored deoxidation by opposing a barrier to the air. Rosenthal<sup>10</sup> suggested the use of lanolin (melting point 42°C.) as a seal, and recently Fildes<sup>11</sup> has stated that oil has practically no effect in preventing the return of oxygen to the medium, while solid paraffins merely delay its passage. In view of the importance of an effective seal, a quantitative comparison of oil and vaseline seemed advisable.

*Experiment 1.*—Twelve tubes, 1.5 by 20 cm., each containing one-sixteenth of the kidney of a 1,600 gm. rabbit, were filled with 1 per cent dextrose broth to a height of 9.5 cm.; 2 drops of sterile 0.5 per cent aqueous methylene blue were added as indicator. Series A consisted of four tubes, not sealed (air control). Series B, four tubes overlaid with 2.5 cc. of paraffin oil. Series C, four tubes overlaid with 2.5 cc. of vaseline. All incubated at 37.5°C.

When observed after 16½ hours the tubes of Series A were decolorized 2 cm. from the bottom of the tube. Tubes of Series B decolorized 3 cm. from bottom. Tubes of Series C completely decolorized.

When observed after 42 hours, the tubes of Series A and B showed an increase of 1 cm. in the height of the decolorized column. Tubes of Series C remained completely decolorized. There was no further increase in the height of the decolorized columns in the tubes of Series A and B.

Repetition with smaller fragments of kidney yielded similar results.

As shown by the point at which a balance was established between the access and diffusion of oxygen and the reducing action of the kidney tissue, the addition of paraffin oil produced only a slight increase in the length of the anaerobic column, the final ratio of the oil-covered to the air-covered tubes being 4:3.

<sup>10</sup> Rosenthal, G., *Compt. rend. Soc. biol.*, 1902, liii, 1132.

<sup>11</sup> Fildes, P., *Med. Research Com., Nat. Health Insurance, Special Rep. Series*, No. 12, 1917, 59.

*Experiment 2.*—Sixteen 20 by 1.5 cm. tubes, filled with broth and methylene blue as before, but without kidney tissue. Series A, four tubes not sealed. Series B, eight tubes sealed with paraffin oil in a column from 0.5 to 4 cm. in height. Series C, four tubes, vaseline seal 1 cm. high. All were placed in a McIntosh and Fildes jar,<sup>9</sup> the oxygen was removed by combustion, and the tubes were incubated at 37.5°C.

After 18 hours Series A was completely decolorized. Series B, 0.5 to 1 cc. of oil, completely decolorized (1.5 to 2.5 cc. decolorized in 42 hours, 3 to 4 cc. in 60 hours). Series C, completely decolorized. Removal from jar and exposure to air caused a prompt return of color in the tubes of Series A and B. Series C remained colorless permanently.

The results of Experiments 1 and 2 are illustrated in Fig. 1.

In the McIntosh and Fildes jar a slight reduction in pressure occurs as a result of the combination of oxygen and hydrogen. The partial vacuum tends mechanically to lower the tension of oxygen dissolved in the medium and so hastens the establishment of strict anaerobic conditions through the action of a reducing agent. When large amounts of paraffin oil are employed, this favorable action is retarded. Indeed, the oil seems to serve as a reservoir for oxygen in solution. Except to prevent evaporation and to maintain anaerobic conditions after removal, the use of any seal appears to be unnecessary in a properly manipulated McIntosh and Fildes jar.

*Experiment 3.*—Six 20 by 1.5 cm. tubes containing 10 cc. of dextrose broth were colored with 1 drop of 1 per cent methylene blue. Series A, two tubes, unsealed. Series B, two tubes, overlaid with 4 cc. of paraffin oil. Series C, two tubes, overlaid with 2.5 cc. of vaseline. All tubes heated in a water bath until color disappeared, then plunged into cool water to bring to room temperature and to solidify the vaseline.

The blue color appeared immediately at the surface of the unsealed tubes and at the surface of the medium in contact with the oil. Streaks of blue carried by convection currents descended to the bottom of the tubes, there to be slowly decolorized again. After 20 minutes a zone of blue was established to a depth of 3.5 cm. in the unsealed tubes and to 3 cm. in the oil-covered tubes. The vaseline-covered tubes remained colorless.

After standing for 72 hours at room temperature the blue zone had descended to a depth of 4.7 cm. in the unsealed tubes and to 4.6 cm. in the oil-sealed tubes. During the following days the unsealed and the oil-sealed tubes came to an equilibrium approximately 6 cm. below the surface of the medium. The vaseline-sealed tubes remained colorless throughout.

Under air or paraffin oil, dextrose broth is unable alone to overcome the diffusion of oxygen, which is hardly retarded by the paraffin oil seal. In the depths of the tube, however, the combined reducing action of broth and kidney is able to establish and maintain an oxygen-free zone, which is slightly higher in the paraffin oil-covered tubes. Under vaseline, on the other hand, owing solely to its solid state at incubator temperature, the access of oxygen is prevented, and the dextrose and kidney tissue in the course of a few hours fix all the oxygen remaining in solution. Other aids to deoxygenation, such as a McIntosh and Fildes jar or the action of heat in reducing the oxygen solubility of the medium and increasing the chemical activity of the alkaline dextrose solution, exert a similar action in hastening the establishment of anaerobic conditions in a culture tube, provided that access of atmospheric oxygen is prevented by an impervious solid seal such as vaseline.

We conclude that the paraffin oil seal extensively used in anaerobic culture work and in gas analysis is practically valueless except to prevent evaporation. The seal itself may contain enough oxygen in solution to defeat the very object which it is used to attain. A layer of vaseline, on the other hand, is an oxygen-resisting seal that materially aids the action of deoxidizing agents in the medium.<sup>12</sup>

#### *Standardization of the Kidney Tissue Component.*

Four functions have been ascribed to the fragment of kidney utilized in the tissue technique: a reducing activity, the formation of a nidus, the contribution of nutritive elements to the medium, and an effect upon the pH concentration by acid production. That the kidney tissue component is an active reducing agent has been well known since 1885 through the researches of Ehrlich and others and is demonstrated anew in Experiment 1 of this communication. But the zone of its action in the culture tube has not been subjected to quantitative estimation.

<sup>12</sup> Hard, inelastic paraffin waxes may be less useful through rupture of their contact with the glass by changes of temperature in the tubes.

Smith,<sup>1</sup> who was the first to advocate the use of tissue fragments, gave no indication of the size to be used. Tarozzi<sup>2</sup> advocated a cube of about 1 cm. Noguchi<sup>13</sup> suggested that the kidney of an adult rabbit should be cut into about ten pieces, each approximately the size of a split chestnut. Other original workers have not defined the tissue component so carefully and the tendency in general has been to use too small a piece, especially for primary cultivations and early transplants.

*Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.*—We tested the reducing effect of pieces of various sizes upon a medium containing methylene blue.

*Experiment 4.*—Two sets of three 20 by 1.5 cm. tubes were prepared, each containing 10 cc. of aerated 1 per cent dextrose broth and 1 drop of 1 per cent aqueous methylene blue in addition to the kidney fragments. The fragments were cut as uniformly as possible from 5 mm. cross-sections through the middle of the kidneys from an 1,800 gm. rabbit. In Set 1 a single piece of kidney was used. Tube A contained one-eighth, Tube B one-fourth, and Tube C one-half of a 5 mm. cross-section. In Set 2 the pieces were cut into eighths of the original cross-section. Tubes A, B, and C were the same as the corresponding tubes of Set 1, except that Tube B contained two-eighths and Tube C four-eighths of a cross-section in two and four separate fragments. No seal. Incubated at 37.5°C.

In Tubes B and C of each series decolorization began in a few moments around the kidney tissue at the bottom of the tube. Later results are shown in Table I and are illustrated in Fig. 2.

After 42 hours the tubes were removed from the incubator and allowed to stand at room temperature. After 20 hours at about 21°C. the level of decolorization had dropped 0.5 to 1 cm. in each tube.

TABLE I.

*Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.*

Set No.	Duration of incubation.	Height of decolorized column from bottom of tube.		
		Tube A; small fragment of kidney.	Tube B; twice as much as Tube A.	Tube C; four times as much as Tube A.
	<i>hrs.</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>
1	16	0.4	2.3	3.0
2	16	1.0	1.8	3.0
1	42	1.7	3.5	4.4
2	42	1.8	3.4	4.4

<sup>13</sup> Noguchi, H., *Münch. med. Woch.*, 1912, lix, 1937.



Different amounts of kidney tissue have different reducing effects. The larger the amount, the greater is the reduction. Small pieces are of little value for producing an anaerobic zone. Very large pieces do not produce a proportionately large zone of reduction. With 0.6 to 0.8 gm. of kidney tissue a zone sufficient for practical purposes is obtained.<sup>14</sup> The tissue may be used in one fragment or in several. The balance between the penetration of oxygen from the surface into the medium and the reducing activity of the tissue occurs at a level determined by the temperature, other things being equal. The higher the temperature, up to 37°C., the less is the oxygen solubility and the greater the reduction.

*Nature of the Reducing Substances in the Kidney Tissue.*—Kidney tissue in unsealed tubes such as those of Experiment 4 exposed to the air at room temperature will maintain an anaerobic zone at the bottom of the tube for a period of weeks or months. It is not conceivable that the reduction is dependent on the maintenance of the activity of living cells. The question arises then, as to the nature of the substance responsible for the reduction. In considering this question, which properly belongs to physiology and biochemistry, we were soon led away from the subject in hand. Preliminary experiments indicated that the activity still resided in filtered extracts of kidney tissue, and that the reducing substance concerned is relatively heat-stable, so that boiled, or even autoclaved kidney is not entirely without effect, but other considerations make it seem unprofitable to attempt to modify the use of fragments of fresh sterile kidney tissue in this anaerobic technique. Thunberg<sup>15</sup> refers to former researches on the reducing activity of tissues and reports his evidence of the enzymotic nature of the reaction.

Before the reducing activity of fresh tissue in anaerobic cultivation was generally recognized, it was suggested that the tissue fragment might act in an obscure and passive manner as a focus for bacterial multiplication. The demonstration of a favorable reaction on the

<sup>14</sup> The kidneys of a medium sized rabbit, 1,400 to 1,700 gm., weigh about 6 to 6.5 gm. Eight to ten fragments may be obtained from each kidney. The large kidneys of full grown rabbits yield twelve to sixteen fragments of proper size.

<sup>15</sup> Thunberg, T., *Skandin. Arch. Physiol.*, 1918, xxxv, 163.

surrounding medium made such an assumption unnecessary. Some investigators, however, acting on the earlier hypothesis, have advocated the use of small pieces of inorganic substances, asbestos wool or iron tacks, for example,<sup>16</sup> as a nidus for anaerobic growth.

Our experiments with these substances in the presence of methylene blue need not be elaborated here. They indicate that washed asbestos wool has no effect in promoting an anaerobic zone. Indeed, in a feeble reducing medium the decolorization of the methylene blue was somewhat retarded. The oxidation of iron tacks, on the other hand, soon reduced the dye in their vicinity. It seems clear that the production of an anaerobic condition depends upon a chemical reaction rather than upon the presence of inert material.

#### *Nature of the Culture Medium.*

*Presence of a Reducing Substance.*—Methylene blue serves as an indicator of the presence or absence of free oxygen only in the presence of a reducing substance. For this reason it is not an accurate index for the removal of oxygen by physical means. Our observations led us to conclude, however, that complete deoxygenation is only very slowly accomplished by the diffusion of oxygen in solution into an oxygen-free atmosphere such as is provided by the method of Buchner<sup>17</sup> or that of McIntosh and Fildes.<sup>9</sup>

In a recent communication Barber<sup>18</sup> indicates that some strict anaerobes may be destroyed even by short exposures—less than an hour—to atmospheric oxygen. Under certain conditions, therefore, it may be important to attain strictly anaerobic conditions in the culture tube in the shortest possible time after inoculation or else to inoculate an already deoxygenated medium.<sup>19</sup> Among the aids to such a procedure is the addition of an active reducing agent (kidney tissue, dextrose, peptone) to the culture medium.

Culture media in general may be divided into two classes, those which contain an active reducing substance, and those which are prac-

<sup>16</sup> Douglas, S. R., Fleming, A., and Colebrook, L., *Lancet*, 1917, ii, 530.

<sup>17</sup> Buchner, H., *Centr. Bakt.*, 1888, iv, 149.

<sup>18</sup> Barber, M. A., *J. Exp. Med.*, 1920, xxxii, 295.

<sup>19</sup> Tarozzi<sup>2</sup> and Noguchi<sup>5</sup> undoubtedly obtained such a condition by preliminary incubation of tissue media to insure sterility.

tically inert. Artificial media containing dextrose or peptone belong in the first category. Ascitic fluid and dilute serum, widely used in anaerobic culture work, belong in the second class. Although small amounts of a copper-reducing substance may be demonstrated in these liquids, their deoxygenating action is relatively slight, and hardly to be considered of practical value. Thus, while kidney tissue was able to decolorize 10 cc. of ascitic fluid containing methylene blue under a vaseline seal in 10 days, similar tubes of ascitic fluid, without kidney tissue, still retained a pale blue color after 6 weeks observation.

The efficacy of dextrose as a reducing agent in alkaline solution suggested its addition to the ascitic fluid medium. Combinations of 0.1 to 2 per cent of dextrose were made by the addition of 10 per cent dextrose solution in isotonic saline solution to the ascitic fluid. Vaseline-sealed tubes containing ascitic fluid and methylene blue and 0.1 per cent dextrose were almost decolorized in 7 days. The larger amounts of dextrose up to 2 per cent, with the aid of a McIntosh and Fildes jar, decolorized the ascitic fluid in 5 to 6 days. Dextrose peptone broth was even more efficacious. One part of 1 per cent dextrose peptone broth with two parts of ascitic fluid decolorized methylene blue under a vaseline seal in less than 42 hours.

In the absence of a reducing agent tubes of ascitic fluid from which air is rigidly excluded may not become decolorized over a period of weeks. The addition of small amounts of dextrose, or the presence of kidney tissue renders ascitic fluid medium oxygen-free in a relatively short time. It should be understood that dextrose here is being considered solely as a reducing agent. Other effects—change of pH concentration through bacterial action, gas formation, etc.—may make its addition undesirable in certain instances.

*Physical State of the Medium.*—A second character of culture medium which influences the establishment of anaerobic conditions is its physical state. Fluid media suffer the disadvantages of ready diffusion of oxygen throughout the tube, of the development of convection currents on even slight changes of temperature, and of the transfer of oxygen to the depths of the tube by any agitation. These phenomena are easily observed in air or oil-covered tubes showing a decolorized zone at the bottom.

Semisolid media, formed by the addition of small amounts of agar, are not subject to these influences to the same extent. It is not surprising, therefore, to find that anaerobic conditions are more readily maintained in the depths of a semisolid culture tube. Thus, the effect of different amounts of agar on the diffusion of oxygen through dextrose broth was tested.

*Experiment 5.*—Tubes were filled with 1 per cent dextrose peptone broth and 2 per cent dextrose agar in proportions to give percentages of agar from 0 to 0.5 in volumes of 10 cc. The mixtures were colored with methylene blue and heated in the water bath to complete decolorization. They were then plunged into water to solidify the agar and incubated at 37°C. The diffusion of oxygen from the surface downward was measured in all the tubes by the advance of the returning color. After 5 and 24 hours the blue zones had descended as shown in Table II, where they remained during subsequent observations.

TABLE II.  
*Penetration of Oxygen into Semisolid Medium.*

Tube No.	Amount of agar.	Penetration of oxygen from surface of medium.		
		After 5 hrs.	After 24 hrs.	After 48 hrs.
	<i>per cent</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>
1	0	1.0	4.0	Complete.
2	0.02	1.0	1.5	3.3
3	0.04	0.9	1.2	2.2
4	0.06	0.9	1.1	1.3
5	0.1	0.8	1.0	1.1
6	0.2	0.8	1.0	1.1
7	0.5	0.7	0.9	1.0

Even so small an amount as 0.02 per cent of agar may inhibit the diffusion of oxygen to the depths of a culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5 per cent of agar was employed.

A more striking experiment is afforded by a comparison of the reducing effect of kidney tissue in peptone broth and in the same broth made semisolid by the addition of 0.25 per cent agar.

*Experiment 6.*—Series A; tubes contained a 9 cm. column of plain broth. Series B; the same plus kidney tissue. Series C; tubes contained a 9 cm. column of semisolid medium (peptone broth plus 0.25 per cent agar). Series D; the same plus kidney tissue. All were colored with methylene blue and were incubated unsealed at 37.5°C. Table III shows the height of the colorless zones which developed from the bottom of the tube in each instance.

TABLE III.

*Development of Anaerobic Conditions by the Action of Kidney Tissue in Fluid and Semisolid Medium.*

Medium.	Zone of decolorization measured from bottom of tube.		
	After 1 day.	After 2 days.	After 6 days.
	cm.	cm.	cm.
Series A; plain broth without kidney tissue.....	0	0	0
“ B; “ “ with kidney tissue.....	1.5	2.0	1.4
“ C; semisolid medium without kidney tissue.....	7.4	7.3	7.3
“ D; “ “ with kidney tissue.....	7.0	7.3	7.3

Besides showing that oxygen penetrates slowly into semisolid media this experiment demonstrates the reducing power of peptone (Series C). Comparison of Series C with Series D suggests that the reducing power of kidney tissue in a semisolid medium is confined to the zone surrounding the tissue, since its effect did not extend further than that of the peptone broth alone.

This observation is confirmed by the following experiment with a medium of much less reducing power, so that the kidney tissue alone was responsible for the production of an anaerobic zone.

*Experiment 7.*—Series A, two tubes, each containing one-tenth of a medium sized kidney, 7.5 cc. of dilute rabbit serum, 2.5 cc. of 2 per cent plain agar, and 1 drop of methylene blue, making a 0.5 per cent semisolid medium. Series B, two tubes, each containing the same ingredients, except that 2.5 cc. of plain broth were substituted for the agar, making a fluid control. Control tubes of each medium, without kidney tissue, were also set up. Vaseline seals. Tubes incubated at 37.5°C. for 24 hours. The tubes of Series A showed a decolorized zone 1.7 and 1.7 cm. high respectively, sharply demarcated from the deep blue agar above. Control, without kidney, deep blue. The tubes of Series B were decolorized 2.4 and 2.3 cm. from the bottom, respectively, shading off gradually to a deep blue above. Control, without kidney, deep blue. After 48 hours the semisolid tubes showed a clear-cut colorless zone, 2.7 and 2.6 cm. high; agar deep blue above. Control

deep blue. The fluid tubes showed a colorless zone approximately 4 to 4.5 cm. high, shading off so gradually that its limits were difficult to determine. The fluid above and the control were light blue.

From these experiments a semisolid medium is seen to favor the establishment of anaerobic conditions by the exclusion of oxygen from the depths of the tube. Unless a reducing agent is diffused through the medium, however, this advantage is offset by a restriction of the reducing power of the kidney tissue to the immediate vicinity of the tissue fragment. These observations may explain in part the difficulty often encountered in growing strict anaerobes in semisolid medium, especially in initial cultivation.

*Length of the Column of Medium as an Aid in Deoxygenation.*—It has become the standard practice in anaerobic cultivation by the tissue method to use 12 to 15 cc. of fluid, or a column 10 to 12 cm. in height in a long narrow culture tube 20 by 1.5 cm. Anaerobiosis in the depths of the tube would seem to be favored by the restricted surface exposed under paraffin oil and by the length of the column of liquid.

It was found in the present study that in a reducing medium such as dextrose broth a column length of from 8 to 16 cm., overlaid with paraffin oil, favors the decolorization of methylene blue, which occurs first at the bottom of the tube and gradually extends toward the surface. Under vaseline, on the other hand, columns of different lengths decolorize with equal rapidity. In dextrose broth the reducing action proceeds throughout the tube. When it is more localized, as with kidney tissue in a less active medium (plain broth), the effect is somewhat different.

*Experiment 8.*—Three sets of tubes of plain broth colored with methylene blue, each containing one-sixteenth of a kidney of a large rabbit, the broth columns being 2, 4, 6, 8, 10, and 12 cm. long. Series A, unsealed; Series B, overlaid with 2 cm. of oil; Series C, overlaid with 1 cm. of vaseline. Control tubes of broth without kidney, column 8 cm. long, unsealed and also with oil and vaseline seals. Incubated at 37.5°C. In 16 hours decolorization had proceeded as shown in Table IV.

When oxygen is excluded (Series C) the fragment of kidney tissue is able to produce an anaerobic zone 2.1 cm. high in 16 hours. That

it did not do so in the 2, 4, and 6 cm. tubes of Series A and B under air or oil is due to the penetration of oxygen. A column of fluid 8, 10, or 12 cm. long maintained at a constant temperature without agitation practically serves as a seal for the lower levels. But the same object is accomplished by a vaseline seal with considerable saving in medium; 7 or 8 cm. of the medium suffice.

TABLE IV.

*Effect of the Height of the Medium Column on the Anaerobic Zone Produced by Kidney Tissue.*

Series.	Height of decolorized column from bottom of tube after 16 hrs.						Control.
	Length of fluid column.						
	2 cm.	4 cm.	6 cm.	8 cm.	10 cm.	12 cm.	8 cm.
	cm.	cm.	cm.	cm.	cm.	cm.	
A	1.0	1.5	1.8	2.2	2.1	2.1	Unchanged.
B	1.3	1.8	1.9	2.1	2.0	2.1	"
C	2.0	2.1	2.0	2.1	2.1	2.1	"

*Effect of Reducing Agents on the pH of the Medium.*

Although this study deals only with the deoxygenation of culture media, it is necessary to know whether the reducing agents employed have any effect upon other essential factors in anaerobic cultivation; for example, the hydrogen ion concentration of the medium. Accordingly, tubes were filled with ascitic fluid or with ascitic fluid and dextrose peptone broth, with or without kidney tissue. To each tube 5 drops of phenol red were added and the tubes incubated at 37.5°C. under a vaseline seal. When observed at intervals during the following days and weeks, the tubes without kidney tissue showed no change from the original hydrogen ion concentration of 8 +. On the other hand, the kidney tissue in 20 hours had produced a clear yellow zone approximately 1.5 cm. from the bottom of the tube. The effect was the same in ascitic fluid and in the mixture of ascitic fluid and broth. The next day and thereafter, all the tubes containing kidney tissue showed a gradation from purplish pink (pH 7.8 to 8) at the surface downward in the acid direction to a clear yellow in the middle and lower portions of the column of medium. This color

gradually became diffused throughout the tubes, bringing the medium to an orange-yellow comparable to pH 7.4 on the phenol red scale.

Ascitic fluid itself is usually alkaline, showing a pH 7.8 to 8+. This alkalinity is progressive if the fluid is allowed to stand exposed to the air but may be retarded by a layer of paraffin oil. From the foregoing experiment it is seen that the alkalinity, once established, remains unaltered for weeks under a vaseline seal. Dextrose and peptone do not in themselves produce acid and thus change the hydrogen ion concentrations. Only when they are split by bacterial activity do they have this effect. Kidney tissue produces acids; a large fragment (0.8 gm.) changes the hydrogen ion concentration in its immediate vicinity to about pH 7. This acid becomes gradually diffused throughout the medium and may ultimately bring the entire column of medium to a favorable hydrogen ion concentration (pH 7.4). The final concentration, then, depends on the ratio of the original alkalinity of the ascitic fluid to the acid production of the kidney.

#### SUMMARY AND CONCLUSIONS.

This study was undertaken with the object of determining the part played by the several component elements of the tissue method of anaerobic cultivation in the establishment of anaerobic conditions in the culture tube. Data have been presented to show the suitability of methylene blue as an indicator of reduction processes in culture media by which the removal of the last traces of oxygen may be demonstrated. With methylene blue as the indicator, the elements subjected to experiment included the choice of a seal for culture tubes, the activity and requisite size of the kidney tissue fragment, the chemical and physical characters of the medium which promote or retard deoxygenation, the length of the column of medium, and the advantages of external aids such as the McIntosh and Fildes anaerobic jar.

As a result of our experiments, we have come to the following conclusions:

1. Liquid paraffin oil, used extensively as a seal for anaerobic cultures and in gas analysis, has very little value in inhibiting the access of oxygen. Solid vaseline, on the other hand, forms an effective



oxygen-resisting seal. The difference is due to the physical states of the substances at incubator temperature.

2. Fresh kidney tissue is an active reducing agent and quickly decolorizes methylene blue in its vicinity. The reducing effect of fresh kidney tissue is relative to the amount used. As a reducing agent, at least 0.6 gm. per tube is required for the establishment of an adequate oxygen-free zone.

3. Culture media may be classified as reducing or non-reducing. Those containing dextrose or peptone in a faintly alkaline solution belong to the former class. Ascitic fluid and dilute serum belong to the latter class, for their content of reducing substances is practically insignificant. For the prompt establishment of strictly anaerobic conditions these media require the addition of reducing substances such as dextrose, peptone, or kidney tissue aided by an effective seal or an anaerobic jar.

4. Semisolid media effectively inhibit the penetration of oxygen to the depths of the tube, but they likewise limit the diffusion of reducing substances and presumably of nutrient substances from imbedded kidney tissue.

5. The length of the column of medium is of minor importance under a vaseline seal.

We clearly recognize the impracticability of standardizing a biological technique which by its very nature must be subject to wide modifications for special purposes. Such variations from a standard are especially necessary in the search for unknown organisms, and in work with hitherto uncultivated microbes in which the tissue technique has been successfully applied by Noguchi.

We wish, therefore, to present the results of our studies simply as guides in the variation and control of the elements examined and to make certain suggestions relative to the establishment of strictly anaerobic conditions in the culture tube. The numerous other factors of equal importance which must be taken into account—hydrogen ion concentration, source and character of nutritive elements, temperature, time, etc.—are outside the limited scope of the present report.

For the establishment of strictly anaerobic conditions in the culture tube, we would suggest (1) the substitution of solid vaseline for liquid paraffin oil as an oxygen-resisting seal; (2) the use of large pieces

of fresh kidney, the standard size to be upwards of 0.6 gm. unless other reducing substances are present in the medium; (3) the addition of peptone or dextrose or both in the form of peptone dextrose broth in fractional percentages to non-reducing media such as ascitic fluid or serum to aid in the prompt establishment of anaerobic conditions; (4) the use of the McIntosh and Fildes jar as a further aid to the prompt deoxygenation of the medium; (5) for reasons of economy the use of smaller amounts of culture medium, for example, 7 to 8 cc., under a vaseline seal; and (6) in dealing with anaerobes which may be injured by exposure to oxygen it might be advisable to prepare the medium a day or two in advance and to incubate it under a vaseline seal so that sterility is assured and the anaerobic conditions are already established when inoculation is made. The infected material is then introduced with a capillary pipette in the vicinity of the kidney tissue and the seal restored by gentle heating to melt a portion of the superposed vaseline.

#### EXPLANATION OF PLATE 2.

FIG. 1. A comparison of paraffin oil and vaseline as oxygen-resisting seals. Sample tubes from Experiments 1 and 2. Dextrose broth and methylene blue without and with kidney tissue, unsealed and under paraffin oil and vaseline. Tubes 1, 3, and 5, without kidney, were deoxygenated by the dextrose broth in a McIntosh and Fildes jar. On removal from the jar the color has returned in the unsealed and oil-covered tubes (Nos. 1 and 3), denoting the penetration of oxygen into the medium. The vaseline-covered tube (No. 5) remains colorless.

Tubes 2, 4, and 6, with kidney, show its reducing effect in the depths of the tube. The unsealed broth (Tube 2) shows almost as extensive a zone of decolorization as the oil-covered broth (Tube 4). Aided by complete exclusion of oxygen, the kidney and dextrose broth have completed the deoxygenation of the vaseline-covered tube (No. 6).

FIG. 2. A comparison of the deoxidizing value of kidney tissue fragments of different size. *A*, a fragment of the size ordinarily employed. *B*, a fragment twice the size of *A*. *C*, a fragment four times the size of *A*. The establishment of an oxygen-free zone is denoted by the decolorization of the medium.

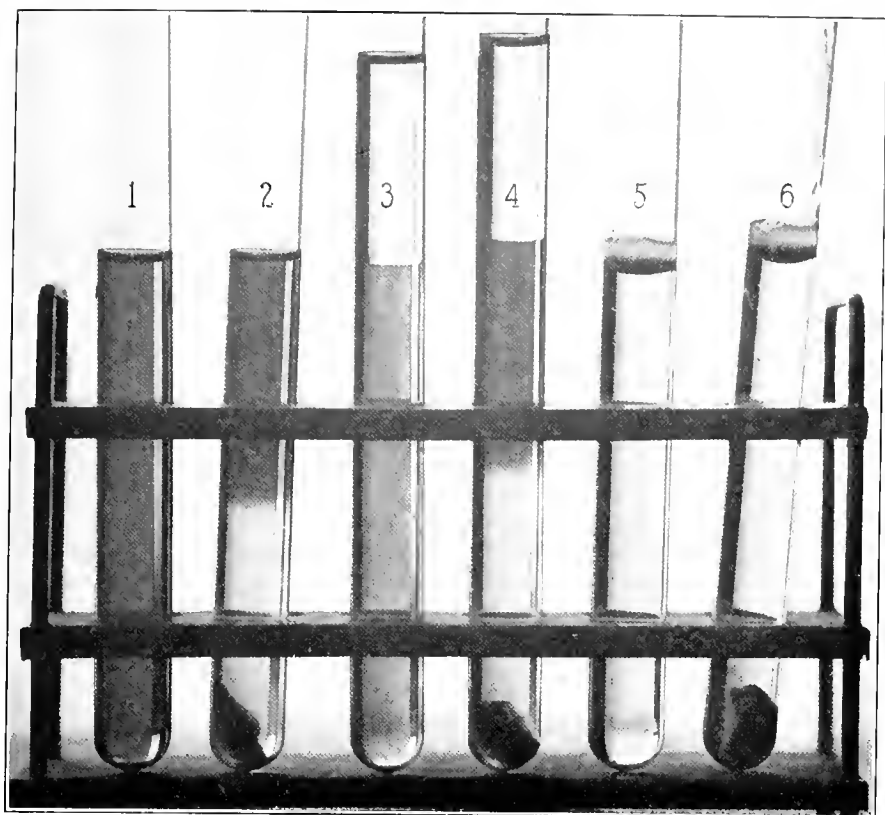


FIG. 1.

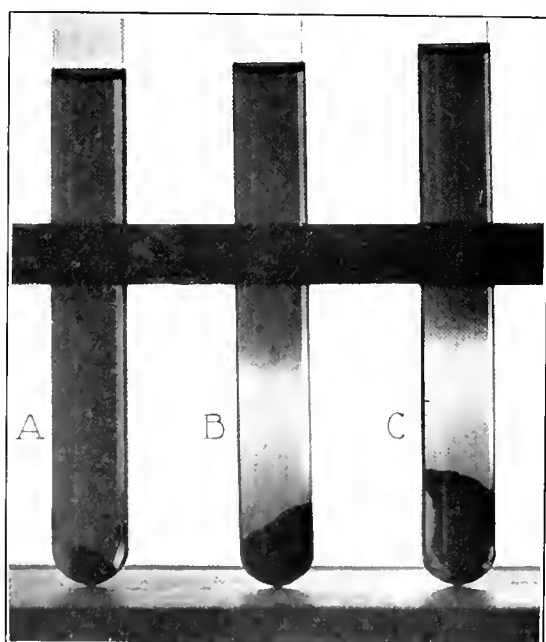


FIG. 2.

(Gates and Olitsky: Factors influencing anaerobiosis.)



## PRESERVATION OF STOCK CULTURES OF BACTERIA BY FREEZING AND DRYING.\*

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(Received for publication, June 29, 1920.)

The object of this communication is to present the results of the prolonged preservation of stock cultures of bacteria after freezing and drying, and to describe the method used.

The benefit of a method that will assure the preservation of cultures of bacteria without frequent manipulation is self-evident. As type organisms are isolated or identified it is desirable to keep the original state of virulence or to retain their cultural characteristics. In addition to the danger of contamination from frequent manipulation, there is the well known tendency for many bacteria to lose their virulence or other biological characteristics from repeated transfers on artificial media. During the course of many investigations it is often of advantage to keep all the bacteria isolated until there is time for more detailed study. A method that will permit the shipping of cultures from one city or country to another without the bulk of culture media, or without danger of breakage or death of the bacteria, has obvious advantages.

The principle of keeping bacteria in a dormant state by desiccation after freezing has been known for several years. Shackell (1) was one of the first to call attention to its advantages. He showed, first, that after freezing, substances such as tissue could be uniformly dried; second, that serum retained its complement and antibody activity unaltered for weeks; and third, that rabies virus in rabbit brains did not lose its virulence when kept in this manner. Hammer (2) dried bacteria previously frozen on strips of filter paper, and kept them from 54 to 57 days. Controls, dried without freezing, were killed immediately. Shattock and Dudgeon (3) showed that organisms dried on charcoal without freezing were usually killed in from 4 to 40 days. *B. pyocyaneus*, however, lived for at least 7

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\* Read before the American and Canadian Section of the International Association of Medical Museums, New York, N. Y., April 1, 1920.

months. Macfadyen (4) and Macfadyen and Rowland (5) demonstrated that practically all bacteria as well as yeast could be frozen and reduced to a temperature of liquid hydrogen, *i.e.*  $-252^{\circ}\text{C}.$ , and later recovered in a viable and unchanged condition. Rogers (6) finally applied the principle of freezing and drying on a large scale so that mass cultures of lactic acid-forming bacilli could be preserved for commercial purposes. He suggested the use of this method for the preservation of stock cultures for the laboratory.

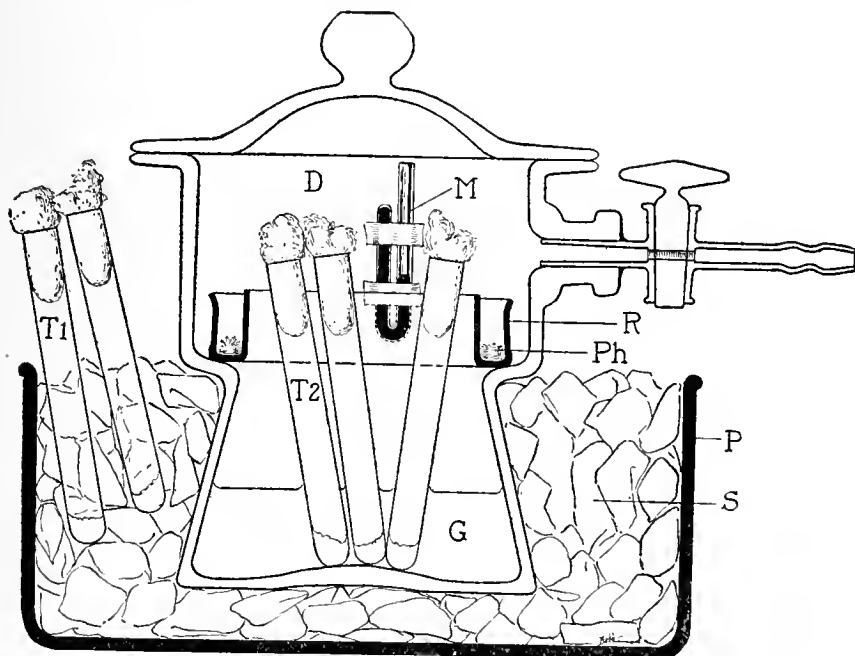
For the past 6 years we have been using this method in the preservation of stock cultures of streptococci and pneumococci. Recently upon attempting to recover these bacteria it was found that if they were originally prepared under proper conditions they were viable after a period of from 2 to 4 years. Comparison of the tubes from which the organisms could not be recovered, with those from which they were easily recovered, showed that the physical state of the dried material had much to do with the viability of the organisms. For example, of twenty-two tubes in which it was possible to recover bacteria, nineteen were in a condition of "dry foam" and three in a condition of "flaky gum." Among eleven with which failure occurred, nine were distinctly gummy, one showed a "flaky gum," and one a "dried foam." This physical state with the accompanying success or failure was present immediately after the organisms were dried or after 4 years. The results showed that it was necessary to maintain the frozen state until drying was complete; this led to the devising of the present method in which the tubes are immersed in glycerol which acts as a medium for the conduction of cold from a salt-ice mixture.

#### *Method.*

*Apparatus (Text-Fig. 1).*—A desiccator (*D*) is chosen with broad opposing surfaces on cover and stop-cock. It is usually necessary to reground all opposing glass surfaces. On the inner side of the desiccator is fastened with adhesive plaster a small mercury manometer (*M*) with one open and one closed end. In the upper part of the desiccator is a receptacle (*R*) for the desiccating salt; if phosphorus pentoxide (*P<sub>2</sub>O<sub>5</sub>*) is used this container can be of copper. In the bottom of the desiccator is placed about 4 cm. of commercial glycerol (*G*). A pan (*P*) is provided, large enough to hold the desiccator and

a salt-ice mixture (*S*) which is packed around the latter. It is necessary to have a mechanical pump that will give a vacuum as low as 2 or 3 mm. of mercury; preliminary exhaustion of the larger part of the air can be conveniently carried out with the ordinary water pump.

*Preparation of Cultures.*—The object to be attained is a maximum number of young actively growing forms of the bacteria in a minimum



TEXT-FIG. 1. Diagrammatic cross-section of the apparatus used for freezing and drying bacterial cultures for preservation. *D*, desiccator; *M*, mercury manometer; *R*, copper receptacle for the phosphorus pentoxide (*Ph*); *G*, glycerol in the bottom of the desiccator; *P*, pan for the salt-ice mixture (*S*); *T 1*, position of a tube of culture during freezing; *T 2*, position of a tube of culture during desiccation.

of fluid. It is necessary to have enough fluid to form a small amount of ice. If fluid media are used it is well to grow the organisms for 15 to 18 hours, centrifugalize the culture, and discard most of the supernatant broth. The concentrated bacteria can then be pipetted into a number of small tubes and frozen. With certain bacteria it

is necessary to use the growth from solid media. In this case the growth should be scraped off into a small amount of condensation water or of broth that has been added, and the suspension subsequently pipetted into tubes. We have found it convenient to use small tubes, about 10 cm. long and from 5 to 10 mm. in diameter.

*Manipulation.*—After the culture has been placed in the small tubes as above described the desiccator containing the glycerol is set in the pan containing the salt and ice mixture; the tubes as well are immersed in this mixture and the cultures frozen (position *T 1*, Text-fig. 1). Simultaneously the temperature of the glycerol is reduced to minus 4–6°C. After careful lubrication of the desiccator cover and stop-cock and after the glycerol has reached a proper temperature and the bacterial suspension is well frozen, the tubes are immersed in the glycerol (position *T 2*), the phosphorus pentoxide is put in the copper pan, and the cover placed securely on the desiccator. The air is then exhausted from the desiccator, first with an ordinary water pump and finally with the high vacuum pump, during all this time the desiccator being kept in the salt-ice mixture. When the proper degree of vacuum has been obtained the whole apparatus is placed in the ice box where it is left until desiccation is complete.<sup>1</sup> This time, in our experience, is usually about 12 hours. When it is certain that desiccation is complete the tubes are removed from the desiccator, the cotton stoppers pushed down into them, and melted paraffin is poured in until the tubes are thoroughly sealed. It is usually necessary to paraffin the tubes two or three times in order to insure complete sealing. If it is desired to keep the organisms for many years the tubes may be sealed by melting and fusing the open ends of the tubes. After the tubes have been well labelled, they can be kept at room temperature, preferably in the dark.

The appearance of the properly dried culture is that of a very light, spongy, flaky material. If it is separated from the sides of the tube it can be shaken about and looks like dried lather made from shaving soap.

*Recovery of Organisms.*—The organisms may be recovered in one of several ways. For such bacteria as the cocci, and those of the

<sup>1</sup> If a frigo ice box is available it is advisable to place the desiccator at a temperature below zero.



colon, typhoid, and dysentery group, as well as other organisms that grow easily in broth, after the removal of the stoppers, broth may be added directly to the dried powder and the tubes incubated. Organisms like meningococci or others that grow better on solid media are best recovered by picking up a small amount of the dried material on a platinum loop and smearing it over the surface of suitable media, after it has been moistened in the water of condensation.

Results obtained by this method with various types of organisms are given below.

*Streptococci and Pneumococci.*—Out of fifteen strains of non-hemolytic streptococci kept from 22 to 40 months, all except two showed growth like that of the original culture, with similar fermentation reactions. These two, however, showed similar types of fermentation reactions with all the subcultures recovered; it is therefore probable that the change in fermentation occurred before the organisms were frozen. In the strains which originally showed a moderate degree of virulence, the virulence was maintained after the recovery of the organism. Four strains of hemolytic streptococci have been recovered after a period of from 42 to 51 months. Three strains of pneumococci were preserved in this manner and upon recovery several months later had retained their original virulence and reaction to type serum. One of the strains that had been accustomed to grow on 75 per cent bile retained this property in the subcultures.

*Meningococci.*—It has been found best to grow meningococci on solid media, scrape off the growth in a small amount of water of condensation, and freeze the resulting suspension. An experiment was performed to determine how small an amount of meningococci might be preserved in this manner; it was found that one loopful suspended in 0.2 cc. of broth or condensation water could be kept for a period of at least 2 months following desiccation. No tests were made after longer intervals. In the recovery of meningococci it has been found much more satisfactory to smear the dried powder upon the surface of freshly prepared blood agar or dextrose serum agar; the addition of blood dextrose broth to the dried powder has yielded more uncertain results. *Bacillus influenzae* is best treated in the same manner as meningococci.

*Other Organisms.*—Typhoid, paratyphoid, and dysentery bacilli have been frozen following growth either in broth or on solid media. They were easily recovered by adding broth to the dried culture.

Other organisms of the bacteria group have not been tested because it is felt that those mentioned above represent the various types. There are few bacteria more delicate and more difficult to maintain in stock cultures than the meningococci and *Bacillus influenzae*. Attempts have been made to preserve the spirochete of relapsing fever recovered from the blood of rats inoculated with these organisms, but the freezing seems to be sufficient to kill completely all the organisms. It is probable that spirochetes in general are not susceptible to preservation in this form, as a number of different strains have been shown to succumb to freezing. No work has been carried out by us with filterable viruses, but Harris and Shackell (7) have shown that the virulence of rabies virus is retained in this manner. Rous<sup>2</sup> has been able to preserve the virus of chicken sarcoma by a similar method for 7 years.

#### SUMMARY.

Attention is called to the fact that bacteria may be preserved for a long time by desiccation in the frozen state. It has been shown that it is necessary to maintain the frozen condition until desiccation is complete; if the fluid melts before the moisture is completely removed, the organisms are killed, probably because of the concentration of the salts upon the surface of the bacteria. By the simple expedient of immersing the tubes of organisms in glycerol contained in a desiccator and subsequently keeping the whole apparatus in a salt-ice mixture until drying is complete, the organisms are easily maintained in the frozen state, and dry properly. Bacteria preserved in this manner retain their cultural, biochemical, and immunological characters for prolonged periods.

<sup>2</sup> Rous, P., personal communication.

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# QUANTITATIVE DISTRIBUTION OF PARTICULATE MATERIAL (MANGANESE DIOXIDE) ADMINISTERED INTRAVENOUSLY TO THE CAT.

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PLATES 3 AND 4.

(Received for publication, June 26, 1920.)

## INTRODUCTION.

The distribution of particulate material introduced intravenously into animals is a subject which has been approached from widely different points of view during the last 75 years. There is, first, a large literature concerning questions of lymph formation, capillary structure, and permeability. Thus, such easily recognized objects as mercuric sulfide particles, milk droplets, etc., have been followed microscopically in their passage from blood to lymph, and numerous efforts have been made to delineate the blood capillaries and lymphatics in organs such as the liver, where their structure is still a matter for debate. With the rise of histological pathology and still more with the growth of bacteriology, similar intravenous injections have been made with the idea of picturing the sites of lodgment of infecting microorganisms. In recent years, under the impetus of investigations by Bouffard (1), Goldmann (2), and others upon the distribution of certain colloidal dyes, many different types of injection in which the individual particles are visible microscopically have been employed. In these instances certain cells which are easily identified by their tendency to take up dye molecules have been shown to possess power to phagocytose visible particulate material as well.

A further cause for the complexity and extent of the literature is found in the uncertainty as to whether bacteria, living or dead, exhibit initial points of lodgment similar to those of inert particulate material such as carbon and mercuric sulfide.

All these questions result in an extensive and confusing literature, which, while it discusses in qualitative terms many important histological and pathological points, is lacking in quantitative data. Thus Ponfick (3), in 1869, injected suspensions of mercuric sulfide intravenously into the frog, dog, rabbit, and guinea pig. The amount of injection used is not made clear. There are no data as to the size of the individual particles, whether they are discrete or tend to agglutinate—in short, nothing which permits judgment upon the percentage of the injected material deposited in any particular organ, not as a result of embolism,

but as a result of fundamental properties on the part of the organ in question. The experiments of Ponfick (3) depend upon histological methods for determination of the distribution of mercuric sulfide, and while such methods must still be used in order to demonstrate the position in any particular organ which deposited material occupies, they are of practically no value in demonstrating the actual extent of deposition for each organ.

With the exception of the work of Voigt (4), who employed colloidal silver and silver iodide injections containing particles of ultramicroscopic size, there is a decided lack of information upon quantitative distribution of particulate material until the period of experiments with bacteria is reached. This deficiency arises from two causes: first, carbon, mercuric sulfide, carmine, fat droplets, etc., are not capable of being determined quantitatively in tissue except with difficulty and at best inexactly; and, secondly, the investigators who have employed such agents have been interested in histological deposition within particular organs and cells rather than in the exact amounts distributed in organs throughout the body.

If we consider, first, the question of inert particulate material such as carbon, we find it generally held that intravenously administered suspensions in the mammal lodge immediately in the liver, spleen, lungs, and bone marrow. In every instance the impression of the investigator as to the amounts reaching each organ is gained from histological preparations, and in order to make these preparations vivid the injected animals are flooded with particles. Under such circumstances one may report that certain groups of cells are extremely active in engulfing particles and may assign them an origin, but the experiment does not lead to conclusions as to the normal activity of the different organs in clearing the circulating blood of foreign particles, nor does it give reliable information as to the extent to which the organs involved engage in this activity.

It is thus clear that important information in regard to the deposition and fate of particulate material in the mammalian body must unfailingly result from the employment of a substance possessing certain attributes. The material must be insoluble in the body fluids and unagglutinated by them, capable of fine subdivision and suspension, and the suspensions employed must have been so prepared as to contain no particles or aggregates of particles capable of blocking capillaries. The material must be visible microscopically, and, most important of all, it must be susceptible to accurate and rapid chemical quantitative estimation even if in extremely high dilution. During the past year Reiman and Minot (5) have developed a method for the quantitative determination of manganese which fulfills the chemical requirements of the situation. In manganese

dioxide, with which this communication deals, and also with other compounds of manganese to be reported upon at a later date, we have found material which meets all the requirements necessary for injection experiments. It has proved possible with manganese dioxide to measure the rate of disappearance from the circulating blood, to determine the amount removed by different organs, and, lastly, to correlate these findings with histological observations which, with this compound, are satisfactory, since the particles are as black and as distinctive as carbon.

#### EXPERIMENTAL.

##### *Manganese Dioxide Suspension.*

The suspensions used for injection have been made from one 5 pound bottle of J. T. Baker's powdered manganese dioxide, chemically pure (84.5 per cent). 10 gm. of manganese dioxide are thoroughly shaken with 200 cc. of distilled water, centrifugalized several times at high speed, and the supernatant liquid is discarded until the particles have been thoroughly washed and the finest material has been largely eliminated.

The next step is to eliminate, by slow centrifugalizing, the larger particles, and in particular the clots composed of aggregates of small particles. This slow centrifugalizing is effected in a 0.4 per cent acacia solution, the acacia serving to hold up the finer particles and thus rendering the selective process of the centrifuge more effective. The acacia used for the purpose is taken from a 10 per cent solution to which 0.1 per cent sodium hydroxide is added until the solution is just alkaline to phenolphthalein. The supernatant liquid from this period of centrifugalization is saved and the residue discarded, since it contains the particles which are large enough to cause embolism.

In the numerous different suspensions used by us during the past year care has been taken not to employ those containing particles or aggregates of particles larger than  $1\mu$ . The experimental data which follow record deposition of manganese dioxide in milligrams of manganese. While the suspensions employed permit enumeration of the individual particles, it is cumbersome to record deposition in

individual organs in terms of number of particles, nor is it essential to do this provided care has been taken to keep the injected material uniform. The actual number of particles in the suspensions per milligram of manganese has varied between 2.5 and 5 billion.

The final suspension, in a 0.4 per cent acacia solution, is sterilized and put up in bottles for future use. Experience has demonstrated the fact that a suspension of manganese dioxide cannot be preserved free from aggregates of particles unless it stands in 0.4 per cent acacia solution and is sterile. Before making an injection the suspension is made slightly saline to the extent of 0.5 per cent sodium chloride, but under no condition should the suspension containing sodium chloride be allowed to stand over a period of hours, since even with acacia present there is a tendency for the particles to aggregate. This tendency is not great, the stabilizing power of even small traces of acacia being pronounced, but since it seems advisable to make injections in which the particles are as nearly absolutely discrete as possible, we emphasize the above precaution.

In order to prove that manganese dioxide is insoluble in blood we attempted to determine whether the compound used could be found to have dissolved when it was exposed to the action of sterile blood at 38°C. Owing to difficulties with filtration and to fear that methods involving precipitation of the blood proteins might also remove traces of soluble manganese, we eventually substituted for blood a solution having the following composition:

	gm.
Sodium chloride.....	8.0
Potassium ".....	0.2
" acid phosphate.....	0.05
Sodium bicarbonate.....	1.0
Calcium chloride.....	0.2
Made up to 1,000 cc. Hydrogen ion concentration = $0.3 \times 10^{-7}$ .	

After shaking up manganese dioxide in this solution the mixture was allowed to stand in an incubator at 38°C. for 6 hours, and then filtered at high pressure. The filter employed consisted of an alundum thimble reenforced by a collodion membrane and fixed in a brass cylinder in such a manner as to be subjected to pressure from an oxygen tank. Filters of this type were shown to permit the



passage quantitatively of soluble manganese salts. The filtrate resulting from the above procedure proved entirely free from manganese, from which fact it seems safe to assume that the manganese dioxide introduced into the mammalian organism, if dissolved by the blood, undergoes this process so slowly as to be negligible.<sup>1</sup>

Finally, we have made *in vitro* agglutination experiments with cat and rabbit serum and manganese dioxide suspensions such as have been used for injection, and after thorough incubation at 38°C. have obtained no evidence that agglutination of particles takes place. It is probable that blood serum could be used to supply protective colloids in making such suspensions instead of acacia.

*Quantitative Determination of Manganese and the Normal Manganese Content of Cat Tissues.*

We have employed the method for manganese determination developed by Reiman and Minot (5), taking the organs from the animals immediately after they have been bled to death, and using not less than 30 gm. specimens when the latter were obtainable.

The second column of Table I gives the average amount of manganese in mg. per 100 gm. of wet tissue taken from fifteen normal cats. In the third column will be found the average value of the same tissue, except the liver, lung, and spleen, taken from twelve cats 1 hour after an injection of manganese dioxide. With the exception of the kidney the two columns are practically identical, and in the case of this organ the difference is extremely small. It is, of course, necessary to omit the liver, lungs, and spleen from this table, since these organs constitute the chief repository for the manganese dioxide injected.

<sup>1</sup> Recent experiments with manganese dioxide indicate that in the presence of organic matter traces of carbonic acid may cause very fine particles to dissolve. While such solution may be of importance for the final elimination of manganese dioxide, it is of negligible consequence in the 1 hour experiments recorded in this paper.

TABLE I.

*Average Manganese Content per 100 Gm. of Wet Tissue in the Organs of Fifteen Normal Cats and of Twelve Cats Injected Intravenously with Manganese Dioxide.*

Organ.	Amount of manganese per 100 gm. of wet tissue.	
	In normal animals.	In animals 1 hr. after injection of manganese dioxide.
	mg.	mg.
Liver.....	0.341	
Lung.....	0.031	
Spleen.....	0.069	
Kidney.....	0.136	0.221
Stomach.....	0.046	0.056
Small intestine.....	0.044	0.060
Colon.....	0.086	0.074
Brain.....	0.035	0.044
Spinal cord.....	0.059	0.041
Muscle.....	0.025	0.024
Urine.....	0	0

*Details of an Injection Experiment.*

Experiments have been performed upon cats anesthetized with urethane, 2 gm. per kilo, and upon normal animals in which a vein for injection has been exposed under local anesthesia. Blood pressure and respirations have been recorded during injection in many experiments under general anesthesia, and as is indicated by Fig. 1 toxic effects have not been observed. The following protocol illustrates the course of a typical experiment.

*Date.*—February 4, 1920.

*Weight of Cat.*—3.7 kilos.

*Anesthesia.*—37 cc. of 25 per cent urethane solution.

*Suspension.*—0.14 mg. of manganese per cc., 0.5 per cent sodium chloride, and 0.4 per cent acacia.

*Injection.*—10 cc. of suspension per kilo.

$$10 \times 3.7 = 37 \text{ cc.}$$

$$37 \times 0.14 = 5.18 \text{ mg. of manganese}$$

*Injection Time.*—Start, 11.21½ a.m.; finish, 11.23½ a.m. Duration 2 minutes. 20 seconds.

Blood samples.			Tracings.	
No.	Time taken.	Length of time after injection.	No.	Time taken.
	<i>a. m.</i>	<i>min.</i>		<i>a. m.</i>
1	11.26½	3	1	11.19
2	11.41½	18	2	11.37
	<i>p. m.</i>			<i>p. m.</i>
3	12.26½	63	3	12.20
4	12.46½	83	4	12.40

*Rate of Disappearance of Manganese Dioxide from the Circulating Blood.*

As a means of indicating the rate at which manganese dioxide particles leave the blood stream, 10 cc. samples of blood were taken at intervals from the carotid artery. As an average procedure four specimens were taken from each cat at varying intervals. Samples of less than 10 cc. would be insufficient to yield positive results if the manganese were still present to the extent of 0.020 mg. per 100 cc. The normal manganese content of cat blood is so low that it is safe to assume that any manganese detectable in a sample of blood not exceeding 25 cc. must be due to the injection. It was found in most cases that taking more than four samples (aggregating 40 cc. of blood) so lowered the blood pressure as to interfere with the circulation. While low blood pressure alone has not altered either the distribution of manganese dioxide or the rate at which it leaves the blood stream, we have not included in these observations experiments in which serious reductions occurred.

Table II displays the data from which certain conclusions may be drawn regarding the rate of elimination. The time interval is indicated by the number of minutes which elapsed between the last drop of the injection and the first drop of blood collected. The actual time required for the process of injection and of blood-taking is insignificant. As an example, the figures in Experiment 5 may be interpreted. The cat weighed 2.4 kilos and was injected with a suspension containing 1.4 mg. of manganese per kilo of cat, or a total of 3.36 mg. of manganese. 3 minutes after the completion of the injection 10 cc. of blood were taken, which upon analysis yielded

0.007 mg. of manganese. The manganese content of the blood is calculated per 100 cc.; therefore in the table the above value appears as 0.070 mg. Again, after an interval of 18 minutes from the time of injection, the blood was found to be negative, which means that 100 cc. contain less than 0.020 mg. of manganese. Examination of Table II indicates that elimination from the circulation is not in any manner dependent upon the dose administered per kilo.

TABLE II.

*Rate of Disappearance of Manganese Dioxide from the Circulating Blood in Normal Cats.*

Experiment No.	Amount of manganese per kilo of cat.	Total amount of manganese injected.	Amount of manganese per 100 cc. of blood.				
			Length of time after injection.				
			3 min.	8 min.	18 min.	63 min.	68 min.
	mg.	mg.					
1	2.45	4.17	0		0		
2	1.40	4.48	0.030		0		
3	1.40	3.22	0.040		0		
4	1.40	3.22	0.046		0		
5	1.40	3.36	0.070		0		
6	2.45	4.17	0.085		0		
7	2.45	6.40	0.093		0		
8	1.40	4.16	0.107		0		
9	0.86	2.75	0.140	0.080	0		
10	1.40	3.92	0.080		0.070	0.040	
11	1.40	5.17	0.138		0.088		
12	2.00	5.20	0.267		0.160		0.088
13	2.00	5.40	0.418		0.244	0.139	

In nine experiments out of the thirteen, the blood is found to be negative after an interval of 18 minutes. In the four in which positive results are found after 18 minutes, though the process of elimination is steady and progressive, the experiment has not been prolonged sufficiently to reach the end-point. Within the limits of injection amounts recorded in Table II elimination is either rapid and complete, or very slow and constant, not depending upon the dose or concentration of the injection but upon some unknown factor in the individual animal.

It has been said that alterations in blood pressure had no effect upon the rate of disappearance of manganese dioxide from the circulating blood. We attempted to alter both distribution and rate of disappearance by a variety of maneuvers but were unsuccessful. Fig. 2 shows an experiment upon a urethanized cat weighing 2.9 kilos in which, after recording the blood pressure, a 25 cc. specimen of blood was taken, and then 35 cc. of a 20 per cent solution of acacia in physiological salt solution were injected. Immediately following this injection 24 cc. of manganese dioxide suspension containing 4.69 mg. of manganese were introduced. The results of analyses upon the blood specimens are given in Table III.

TABLE III.

*Disappearance of Manganese Dioxide from the Circulating Blood Following a Large Injection of Acacia Solution.*

Amount of manganese per kilo of cat, 1.62 mg. Total amount of manganese injected, 4.69 mg.

Length of time after injection.	Amount of manganese per 100 cc. of blood.
<i>min.</i>	<i>mg.</i>
3	0.109
18	0
63	0
83	0
103	0

It is obvious that the high acacia concentration in this animal had no effect in holding particles within the circulating blood. There is complete removal in less than 18 minutes, just as is most often true for normal animals. Analysis of the tissues of this cat showed deposition of manganese dioxide in the organs and in the proportionate amounts characteristic of normal animals, so that neither the existence of acacia in high concentration within the blood vessels nor the very high blood pressure (220 mm. of mercury) in any way affected the result. In another animal under similar conditions of anesthesia the blood pressure was lowered markedly by histamine and remained low during the 1 hour period following manganese dioxide injection. Here again results were obtained which differ in no way from those gained with normal animals.

It is thus shown that two substances, acacia which reduces capillary permeability and histamine which increases it, have no effect either upon the removal from the blood stream or upon the distribution of the particles studied.

When suspensions of manganese dioxide far stronger than those employed in the instances cited in Tables II and III are used, the blood does not become free in 1 hour. In Table IV results are given showing the manganese content of the blood at the end of varying periods of time and following large injections.

TABLE IV.

*Manganese Content of the Circulating Blood Following Large Injections.*

Length of time after injection.	Amount of manganese injected.	Amount of manganese per 100 cc. of blood.
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>
1	40.6	0.480
6	36.1	0.560
18	36.1	0.533
19	36.1	0.245

#### *Distribution of Manganese.*

A series of twenty injection experiments was carried out in order to determine the distribution of manganese throughout the body of the cat after an interval ranging from 63 to 83 minutes. An analysis of the liver, lung, spleen, kidney, stomach, small intestine, colon, brain, spinal cord, muscle, blood, and urine of twelve cats gave evidence that the first three organs were the only tissues affected to any significant degree by an intravenous injection of manganese. Table V shows the results obtained with reference to the liver, lung, and spleen.

The weight of manganese normally existing in a particular organ of an injected cat must be deducted from the weight of manganese recovered from it. For example, a liver taken from an injected cat weighs 87 gm.; the manganese content of normal cat liver is 0.341 mg. per 100 gm. of tissue; 87 gm., therefore, contains  $\left(\frac{87}{100} \times 0.341\right)$  0.297 mg. of manganese; the above liver was found to contain 3.740 mg. of manganese; deduct the normal content (3.740 - 0.297) and

the manganese content of this liver resulting from the injection is 3.443 mg. All the values in Table V have been arrived at in this manner.

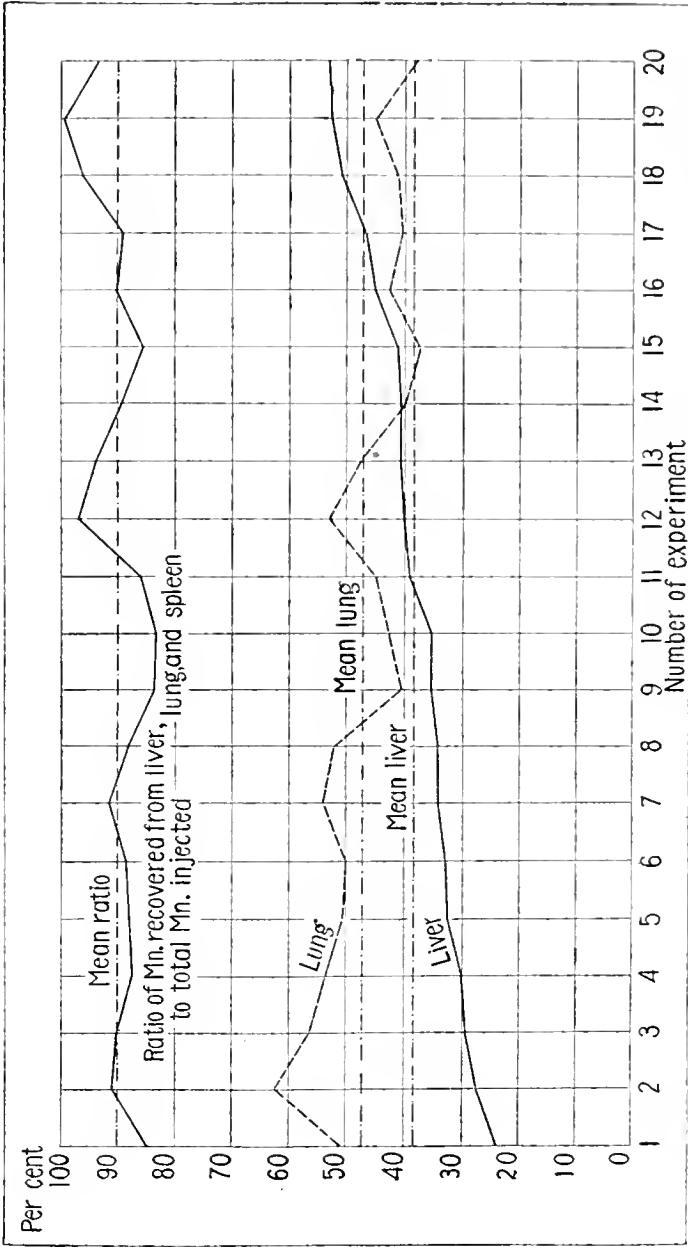
The concentration of the suspension will be seen to vary from 0.100 to 0.245 mg. of manganese per cc. The dose of manganese

TABLE V.

*Distribution of Manganese in the Liver, Lung, Spleen, and Other Organs Following the Intravenous Injection of Manganese Dioxide.*

Experi- ment No.	Amount of manganese per cc. of suspension.	Amount of manganese injected.	Per cent of injected manganese in liver, lung, and spleen.	Per cent of injected manganese in other organs.	Per cent of injected manganese in liver.	Per cent of injected manganese in lung.	Per cent of injected manganese in spleen.
	mg.	mg.					
1	0.100	4.80	85	15	24	51	10
2	0.165	4.96	92	8	27.5	62.5	2
3	0.245	8.36	90	10	29.3	56.4	4.3
4	0.221	4.20	87.3	12.7	30	53.8	3.5
5	0.245	7.54	88	12	32.7	50.7	4.6
6	0.245	8.07	88.5	11.5	33	50	5.5
7	0.187	4.68	91.4	8.6	34.4	54	3
8	0.245	7.53	88	12	34.5	52	1.5
9	0.245	5.48	83.3	16.7	35.5	40.2	7.6
10	0.245	9.80	83.5	16.5	35.5	42.5	5.5
11	0.245	4.17	86	14	39	45	2
12	0.140	3.92	97	3	40	53	4
13	0.245	7.81	93.7	6.3	40.5	47.5	5.7
14	0.245	6.40	89	11	40.5	40	8.5
15	0.100	7.00	85.5	14.5	41	37.5	7
16	0.245	4.17	90	10	45	42.5	2.5
17	0.245	5.39	89	11	46.5	40.5	2
18	0.245	7.28	96	4	51	41	4
19	0.245	6.20	99	1	52.5	45	1.5
20	0.195	6.05	93	7	53	38	2
Average . . . . .			90	10	38.3	47	4.3

administered varies according to the weight of the cat and the concentration of the suspension. Except in Experiments 1 and 15, 10 cc. of suspension were injected per kilo of cat. In Experiments 1 and 15, 20 cc. per kilo of a comparatively weak suspension were injected. Thus, in Experiment 11 a suspension having a concentration of 0.245 mg. of manganese per cc. was injected into a cat weighing



TEXT-FIG. 1. Percentage concentrations of manganese in liver, lungs, and spleen 1 hour after manganese dioxide injection. For the amounts injected see Table V.



1.7 kilos. If 10 cc. per kilo are injected the dose must contain  $(0.245 \times 17)$  4.17 mg. of manganese.

Column 4 gives the per cent of the injected manganese recovered from the liver, lung, and spleen. Column 5 represents the amount of manganese which has escaped the liver, lung, and spleen, and therefore must be deposited in other parts of the body.

Column 6 shows the per cent of the injected manganese recovered from the liver. In Experiment 11, for instance, of the 4.17 mg. of manganese injected into the cat, 1.63 mg. were found in the liver, representing  $\left(\frac{1.63}{4.17}\right)$  39 per cent. Columns 7 and 8 give the same calculations as applied to the lung and spleen respectively. Text-fig. 1 gives graphically the salient facts derived from Table V.

Such variations in the concentration of the suspension as we have employed have had no effect upon the distribution within the organism. It would appear that the work of eliminating the manganese from the blood stream is divided between the liver and the lung in such a manner that they act reciprocally; that is, following injection about 85 per cent of the particles are at once removed by the liver and lung, the lung usually receiving the larger part, but in every instance what is not taken by one of these organs is taken by the other. There is an insignificant amount, 14.7 per cent as an average, in the twenty experiments recorded in Table V, which goes to other organs. The spleen receives a constant slight increment, but the small and variable size of this organ in the cat causes great variation in the amount deposited in it. An average of 4.3 per cent in twenty different spleens represents the activity of this organ. The kidney, intestinal tract, and bone marrow retain manganese dioxide particles in varying and minute amounts, totaling 10 per cent in the experiments recorded in Table V.

#### DISCUSSION.

The results which we have obtained relative to the rate of disappearance of manganese dioxide from the circulating blood are not paralleled by any other work with injections of non-living material.

Hoffmann and Langerhans (6) employed a suspension of mercuric sulfide containing 0.5 gm. per cc. of water. Large amounts of the suspension were injected. In guinea pigs receiving 3.9 gm. of mercuric sulfide, cells containing cinnabar granules were found in the blood 24 days after injection. It is impossible to determine the history of such particles, and the same is often true in more recent work with carbon, carmine, etc.

With injections of microorganisms the case is different. The experiments of Wyssokowitsch (7), who introduced a variety of yeasts and microorganisms intravenously into dogs and rabbits, demonstrate a partial or complete disappearance of these particles from the circulating blood shortly after injection. In two experiments on yeasts a few spores were left at the end of 1 hour, though the injection was enormous. Saprophytic bacteria such as *B. subtilis* disappeared with extreme rapidity and completeness. In one instance when 1 cc. of a concentrated suspension was introduced in the rabbit the blood proved sterile after 15 minutes. With organisms such as *B. anthracis* and *Staphylococcus aureus* the initial rates of removal from the blood stream seem comparable to those obtained with saprophytic organisms, but in these instances there is later a tendency for the appearance in the blood of large numbers of organisms, and this phenomenon is followed by the death of the animal. Wyssokowitsch (7) had no conception of many of the reactions which may be aroused by injected protein material. His results merely indicate the promptitude with which the blood gets rid of very large numbers of living foreign particles. Bull (8) has examined more fully the rate and manner of disappearance of streptococci, pneumococci, and typhoid bacilli from the blood of the rabbit. The manner in which this animal meets such infection varies with the aggressive power of the bacteria employed and the defensive power of the animal, but essentially removal from the blood stream seems to follow the lines already laid down by Wyssokowitsch (7). Thus, with streptococci of low virulence Bull (8) reports an injection into the ear vein of 0.25 cc. per kilo of a bouillon culture of streptococci. After 30 minutes 52 colonies were obtained, following the use of a uniform technique for blood culture. After 2 hours 1 colony was found, after 48 hours 100 colonies were obtained, and after 72 hours the colonies became so numerous as to be uncountable. Later another rabbit injected with the same strain and dose of streptococci, attenuated in virulence as a result of continued cultivation on artificial media, showed sterility of the blood at the end of 2 hours, and this sterility was maintained until 168 hours had passed, when there was a reappearance of streptococci in the blood stream followed shortly by the death of the animal.

With typhoid bacilli Bull (8) presents the following experiment.<sup>2</sup>

"A rabbit weighing 2,000 gm. was given  $\frac{1}{40}$  of a 24 hour agar slant of typhoid bacilli in the ear vein. Blood cultures made from the heart at stated intervals gave the results indicated in Table I."

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<sup>2</sup> Bull, C. G., *J. Exp. Med.*, 1915, xxii, 476.

TABLE I.

Time after injection.	No. of colonies per cc.
<i>min.</i>	
1	10,000,000
2	2,500,000
5	100,000
15	40
20	1

Although we cannot make a direct and final comparison between intravenous bacterial injections and intravenous injections of non-living material such as the manganese dioxide employed in the present study, it seems established that in as far as the immediate removal from the blood stream is concerned the two types of injection are treated similarly by the animal. Furthermore, although the cat differs from other animals in the organs which take foreign material from the blood stream, it does not seem to differ in the rate at which material is removed.

Bull (8), in the case of pneumococci and typhoid bacilli, has related the speed and thoroughness of blood sterilization in the intravenously injected rabbit to an *in vivo* agglutination of the organisms in question by means of immune bodies present in the animal or introduced just prior to injection of the culture employed. He, however, does not make explicit just how this agglutination brings about blood sterilization except in instances in which the blood infection is massive and there is evident blockage of the vessels, particularly in the lungs, by large clumps of organisms. When, however, the bacteria are less numerous, their clumping is not mentioned as an important factor in removal from the blood stream but seems to signal a condition which is followed promptly by phagocytosis. Finally, it is of importance to note that the organs in which the clumped bacteria are chiefly removed are designated by Bull (8) as liver, spleen, and lungs. These are the points at which non-living and non-agglutinated living particles accumulate. If, in Bull's (8) observations, embolism offered an explanation for removal of the organisms from the circulating blood, there is no obvious reason why the capillaries of other organs such as muscle should not take part in the sterilization. That they do not is thus evidence for some specific type of activity in certain organs resulting in bacterial removal from the blood and favored in some way, not understood, by the action of immune bodies in bringing about agglutination.

Kitagawa (9) injected pneumococci unagglutinable by the serum of the rabbit *in vitro* and unagglutinable by the blood of the same animal *in vivo*. The latter fact he proved by isolating samples of infected blood between ligatures placed about the larger blood vessels. He concluded that the disappearance of the

organisms from the blood stream "could not be due to a purely mechanical filtration of agglutinated masses by the tissue capillaries, but that it necessitated some active biological cooperation of the capillary cells" (10).<sup>3</sup>

In the present work the failure to affect the rate of removal of manganese dioxide from the circulating blood and to alter the ratio of distribution in different organs through changes in blood pressure and in concentration of the suspensions employed, points again to the fact that some specific activity on the part of particular cells in certain organs is responsible for removal and that simple mechanical factors are of little or no importance.

Mallory (11) has called attention to the fact that practically all vascular endothelium can become phagocytic. Thus, if blood vessels are injured it can be shown that during the process of wound repair phagocytic cells arise from the proliferating endothelium (12). There has long been a controversy as to whether such cells did not wander into the traumatized area from recognized sites of origin as the milk patches of the omentum. Foot (13) in recent experiments upon this subject found that proliferating vascular endothelium is markedly phagocytic. He discusses the question as to whether connective tissue cells in foci of inflammation may not also contribute phagocytes, but concludes that they do not.

It therefore becomes apparent that phagocytosis is a potential property of vascular endothelium. In certain localities, such as in the capillaries of muscle, this power does not seem to be exhibited except following injury and resulting proliferation. Other regions, notably the liver, spleen, and lung of the cat, possess vascular endothelium which under normal conditions is capable of removing foreign particulate material from the circulating blood. With bacteria there is evidence that the ability of the endothelium to perform this function varies. Manwaring and Coe (10) perfused the livers of normal and immunized rabbits with defibrinated blood to which pneumococci had been added. In the normal rabbit even a dozen passages through the liver reduce the pneumococcic count but slightly, whereas in the animal actively immunized against pneumococci two to five passages sterilize the perfusing blood. This effect is attributed to an opsonin which so alters the pneumococci as to cause them to adhere to the endothelial cells lining the liver capillaries. The same opsonin is practically inoperative in promoting bacterial removal by endothelial cells of extrahepatic tissues.

Hopkins and Parker (14) injected streptococci intravenously in cats and rabbits and found an initial deposition for the cat in the lungs, liver, and spleen with slight lodgment in bone marrow, lymph nodes, muscle, and kidney. The lung of the cat received the largest number of organisms, whereas in the rabbit the

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<sup>3</sup> Kitagawa, K. J., quoted by Manwaring and Coe (10), p. 402.

lung is far less active than the liver. In a subsequent paper Lund, Shaw, and Drinker (15) bring out the same difference between the cat and the rabbit in the handling of manganese dioxide particles. These animals, therefore, exhibit an initial capacity to remove foreign material from the circulating blood which is the same for living and non-living provided the particles in question are of approximately the same size. Hopkins and Parker (14) report that in the rabbit which has been partially immunized against streptococci the lung exhibits an initial power to remove the organisms equal to that seen in normal cats. Here, again, there is evidence of varying activity which in the last analysis seems to reside in the capillary endothelium. While in the case of carbon and starch (16, 17) it has been shown that there are non-specific opsonins which promote phagocytosis, it has never been possible to display variation in the deposition or rate of removal of non-protein material as a result of repeated injections. While, therefore, one may conclude that the initial reaction of the animal against foreign particles is the same, in the case of bacteria subsequent reactions accomplishing removal from the blood stream may be modified as a result of the appearance of immune bodies.

The actual mechanism by which the particles are removed is evidently highly specialized in certain organs. It involves, first, an arrest of the particles as they pass through the capillaries. There is no evidence in our own histological preparations that this arrest is the result of embolism, nor, as has been pointed out, is there evidence in experiments with bacteria that embolism plays an important part. In the case of the lung Hopkins and Parker (14) make the following statement.<sup>4</sup>

"In lung sections of animals killed 10 to 30 minutes after injection it is clear that some of the cocci are held within the leucocytes in the capillaries. It is equally clear that the majority are not located within leucocytes. Many of them lie in large mononuclear cells which are probably the swollen endothelium of the capillaries; others lie in an eosin-staining matrix which may represent a section through endothelial protoplasm, or possibly some substance from the blood deposited about them.

"In order to study this point more accurately, we have etherized and killed cats within 30 minutes after injection, washed the lungs as free as possible by perfusion with salt solution, and then fixed them by injecting Helly's fluid under moderate pressure in order to keep the capillaries distended. In sections fixed in this way the streptococci are not washed from the capillaries; even leucocytes are still present in moderate numbers. Of the mechanism by which the cocci are held, however, we are still in doubt, though many appear to be in endothelial cells."

Foot (13), who made large injections of a carbon suspension in rabbits daily over a considerable period, states that the capillary endothelial cells take up the particles. Slavjansky (18), several days after a heavy intravenous injection of mercuric sulfide in rabbits, found cells containing the substance free in the alveoli.

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<sup>4</sup> Hopkins and Parker (14), p. 11.

He believed, as did Tchistovitch (19), on the basis of similar observations, that these cells were derived from the macrophages of the circulating blood. In all the instances cited the injections were extremely large. Sewell (20) injected a rabbit intravenously with a carbon suspension, the strength of which is not indicated. He, however, filtered it carefully and seems to have avoided the possibility of pulmonary embolism, a contingency from which Foot's (13) experiments would not seem to be free. He found little or no carbon deposited in the rabbit lung and no carbon-containing cells in the alveoli, and contends that certain of the alveolar cells have the property of taking up particles from the air but not from the alveolar structure upon which they rest.

In the present study histological preparations made from cats killed 1 hour after injection of manganese dioxide have shown the material in the following locations in the lung. (1) A very small amount, seen only after protracted search, is still within the capillaries and apparently free. These granules are usually single or in small groups. They do not seem to be attached to any cells, but evidently are not progressing through the lungs, since we have found them so situated when the circulating blood was entirely free from manganese. (2) Particles are found in or on the surface of the capillary endothelial cells. This fraction represents the larger part of the lung content. (3) Groups of particles, not agglutinated, lie within the alveolar septa, particularly at points where septal junctions occur. These points represent the second most important site of the particles. (4) Granules are seen within large lining alveolar cells which occur particularly at points of alveolar junction. Occasionally these cells lie free within the alveoli.

We believe that the initial lodgment of practically all particles in the lung is within or upon the lining capillary endothelium. This deposition of manganese dioxide is very uniform through the different lobes of the lung. As this deposition must express the extent of the capillary bed at the moment of injection, it is a fair assumption that the capillary blood flow is proportionally uniform through the different lung lobes moment by moment.

The function of the liver in removing foreign particles from the blood stream is of greater significance than that of the lung. In the cat we have found a large initial deposition of manganese dioxide in the lungs, but we have been able to show<sup>5</sup> that from the 3rd

<sup>5</sup> Drinker, C. K., and Shaw, L. A., unpublished observations.

hour following injection the material in the lungs begins to diminish, accumulating at first in the liver and from this organ passing into the intestinal canal. Furthermore, as pointed out by Lund, Shaw, and Drinker (15), the lungs of the common laboratory animals, with the exception of the cat, achieve only a slight share of blood sterilization. The capillary bed of the liver differs from that of the lung in that the lining endothelium contains elements, the stellate cells of Kupffer (21), which have long been known to be actively phagocytic. It has also been contended that these capillary walls are incomplete (22), permitting material in the portal system to move directly into the liver cells. Whether or not this is true, it seems certain that the liver cells themselves have little to do with the initial removal of foreign material from the portal blood.

The initial positions of manganese dioxide in the livers of cats injected as in these experiments are as follows: (1) Particles are found free within the capillaries. Just as with the lung, even when the circulating blood of the animal shows no manganese, occasional single particles and groups of particles are found in the capillaries. They are not intracellular and as far as can be seen are completely unattached to the capillary walls. Shipley and Cunningham (23) immersed the omentum of the decerebrate cat in an India ink suspension, and after an interval found many fine, free carbon particles in the liver capillaries. They present evidence that this material is taken up by the omental capillaries and express surprise upon finding it outside cells within the blood channels of the liver. Apparently the particles which we introduced directly into the blood stream became arrested in the same way, but none of the sections has given any evidence of capillary blockage. (2) Large collections of particles are seen in or upon the Kupffer cells. These cells unquestionably account for the great activity of the liver in clearing the circulation of foreign material. The ultimate fate of substances engulfed by them is unknown. There is no evidence which indicates that material once deposited in the liver becomes free in the sense of passing back into the circulation to be deposited elsewhere. Once taken up by the liver, particles may be considered to be on the way toward removal from the body. (3) Occasional granules are found upon the surface of the liver cells. At the end of 1 hour these are very few and repre-

sent an unimportant method of disposal, a point against the theory of the immediate relation of the liver parenchyma to the portal blood.

In the case of the spleen manganese dioxide is found diffusely scattered through the pulp. Many of the particles are free and many have been taken up by large phagocytic cells. As with other types of injection, the Malpighian corpuscles are quite free from particles. The capillary circulation of the spleen is open, and diffuse deposition of material through the pulp is the natural expectation under such mechanical conditions. The presence of phagocytic endothelial cells throughout the pulp assures the seizure of material which drifts into this tissue, but the ultimate fate of particles so phagocytosed is unknown.

In addition to the lungs, liver, and spleen, we have found varying small deposits of manganese dioxide in other organs. The bone marrow remains comparatively free. Bacterial lodgment often occurs in the marrow, which is known to be a reservoir of endothelial phagocytes. In spite of these facts we have found it of little or no importance in clearing the blood of manganese dioxide. The kidney and the intestinal tract in general take the material in varying small amounts in different animals. Often they have remained absolutely negative and at best are of little importance. Muscle, skin, and nervous tissue are invariably free. In this relation it may be noted that while Hopkins and Parker (14) in their studies of streptococcic lodgment found minimal numbers initially removed in muscle, they attribute the eventual serious consequences in their animals to proliferation of these muscle organisms, finding that those removed by the liver, lungs, and spleen have far less chance for growth.

#### CONCLUSIONS.

1. Manganese dioxide suspended in an acacia-sodium chloride solution provides a non-toxic injection which in the present experiments has contained no particles larger than  $1\mu$  and which, when deposited in the body, can be determined quantitatively and seen microscopically.

2. Intravenous injections have been made under precautions which preclude removal from the blood or deposition in organs through simple capillary blockage.



3. In nine experiments out of thirteen the circulating blood contained no manganese after 18 minutes. In the four remaining instances there was a steady slight elimination which was incomplete at the end of 1 hour. Within certain limits the rate of removal from the circulating blood and the sites of deposition in the animal are not influenced by the concentration of the suspension, the blood pressure, or antecedent introduction of acacia or histamine.

4. In the cat amounts of manganese dioxide varying between 9.8 and 3.9 mg. of manganese and containing from 50,000,000,000 to 10,000,000,000 particles, if injected intravenously, permit recovery at the end of 1 hour of 90 per cent of the material in the lungs, liver, and spleen in the following proportions: lungs 47 per cent; liver 38.3 per cent; spleen 4.3 per cent.

5. These experiments, coupled with correlative results by other investigators, make it clear that in certain organs—the lungs, liver, and spleen of the cat—the vascular endothelium possesses phagocytic power rendering the capillaries permeable to particulate material as well as to gases, liquids, and dissolved substances.

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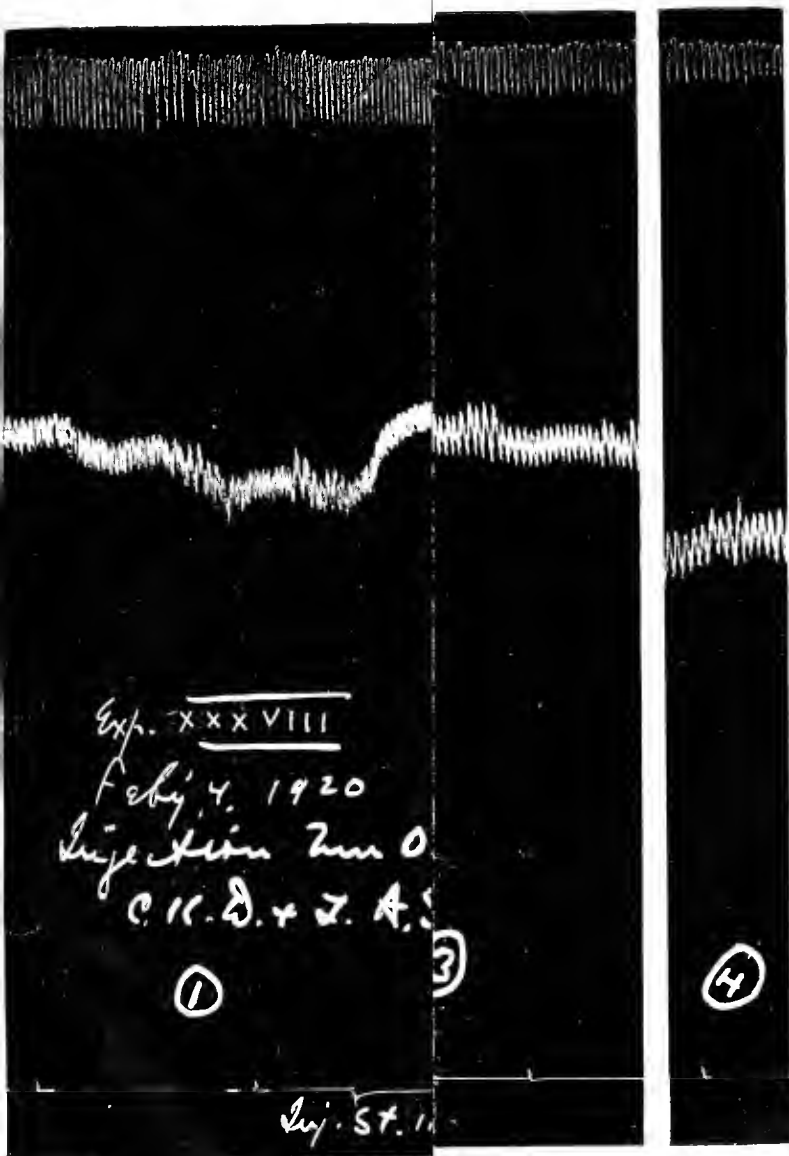
#### EXPLANATION OF PLATES.

##### PLATE 3.

FIG. 1. Injection of manganese dioxide suspension into the femoral vein. Upper curve respiration, downstrokes representing inspiration. Lower curve blood pressure. Base-line 1 minute intervals marking upward; injection signalled downward. Between Tracings 1, 2, 3, and 4, specimens of blood were withdrawn for analysis.

##### PLATE 4.

FIG. 2. Tracing 1 indicates normal blood pressure. Between this tracing and No. 2, 25 cc. of blood were withdrawn from the carotid artery. Tracing 2 indicates first the low blood pressure consequent upon this hemorrhage and then the rise attendant upon acacia solution and manganese dioxide solution injections. Tracings 3, 4, and 5 indicate the blood pressure during the remainder of the experiment. The gradual fall of pressure is due to withdrawal of blood for analysis.



manganese dioxide administered intravenously

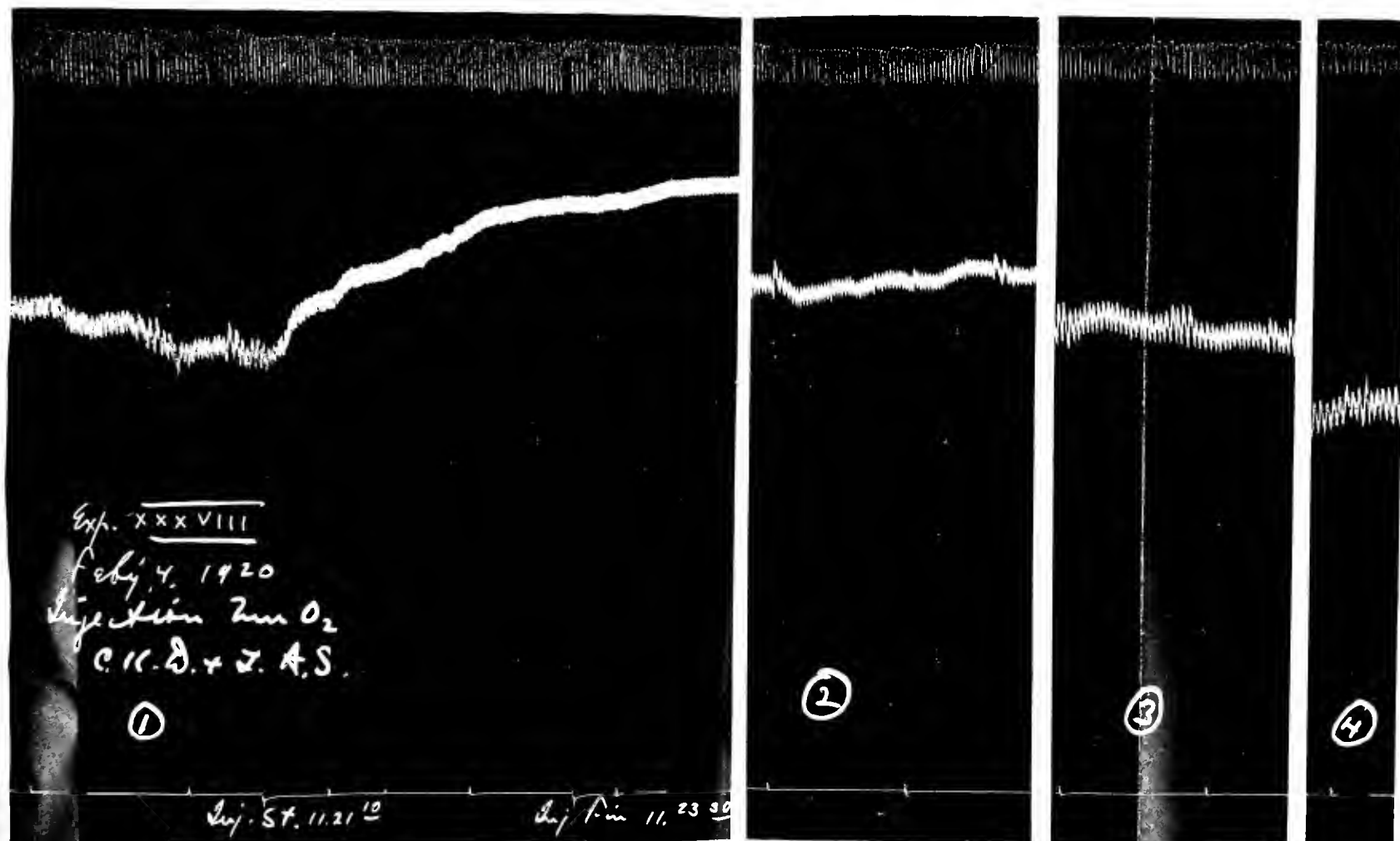


FIG. 1.



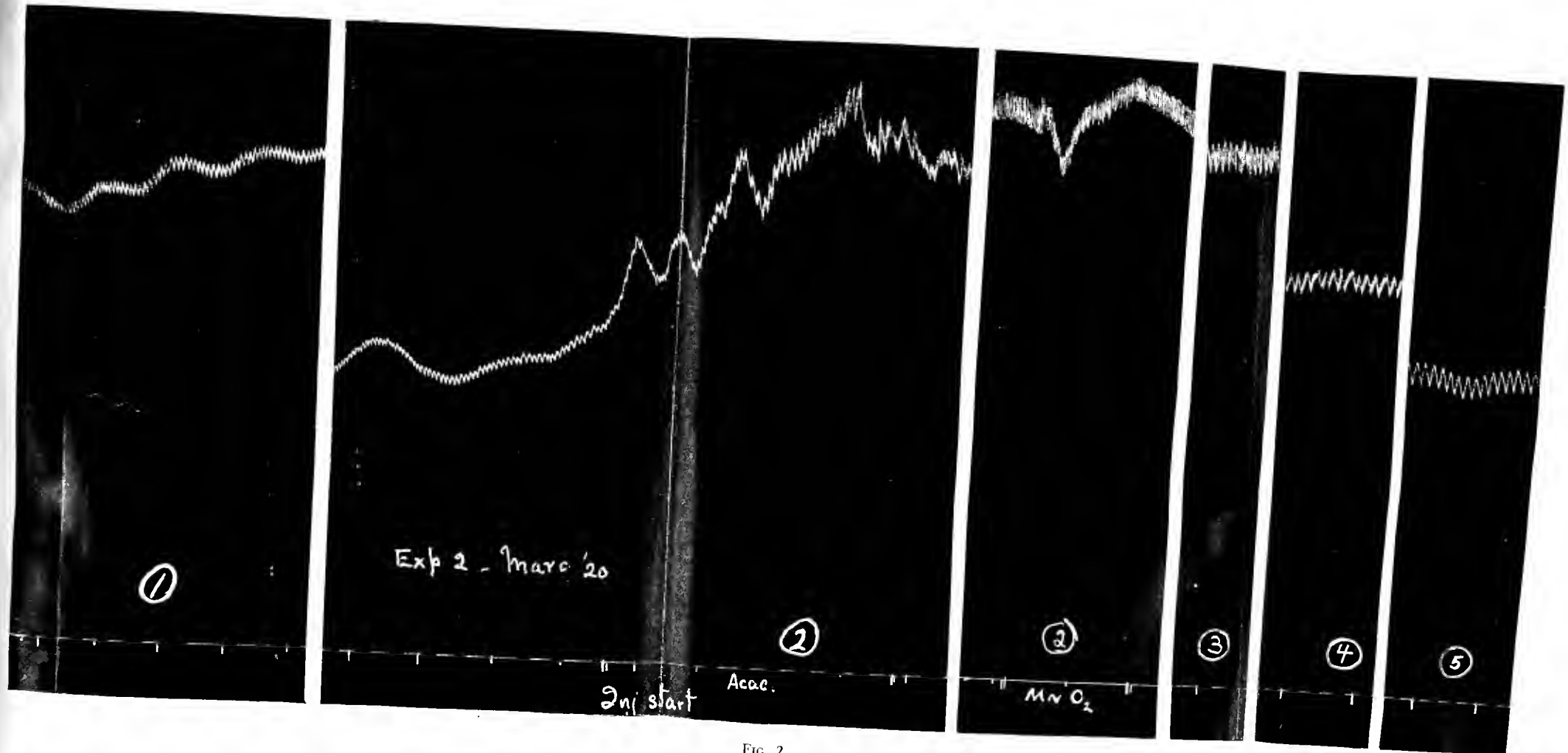


FIG. 2.

(Drinker and Shaw: Manganese dioxide administered intravenously.)

## STUDY OF THE CLASSIFICATION OF MENINGOCOCCI.\*

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(Received for publication, July 9, 1920.)

Work on the classification of meningococci was started in January, 1918, in order to determine whether it was possible to classify by their agglutination and antigenic properties a sufficient number of strains among the cultures at our disposal, so that satisfactory representative cultures could be selected for standardizing serums or immunizing horses. At that time 53 meningococcus cultures were being used in these laboratories in immunizing horses for the production of antimeningococcus serum and for the standardization of the serum by agglutination tests. By the methods of immunization then in use no attention was paid to serological differences, except that the strains of meningococci which showed the least agglutination in the serum of the horses under immunization were injected more frequently than the others. The objection to this procedure is evident, for antibodies may be produced for the strains which show very little agglutination, to the detriment of antibody production for the strains which are more agglutinable. It was thought that a better balance might be obtained by injecting representative cultures in equal amounts, if it was found that a few cultures representative of all or nearly all meningococci could be selected.

The principal methods used to differentiate meningococci into groups have been the agglutination test and the absorption of agglutinin test. Complement fixation and virulence tests have been of questionable value owing to difficulty of technique and to the slight or variable pathogenicity of the meningococcus for small laboratory animals.

\* Presented at the 47th annual meeting of the American Public Health Association, New Orleans, La., October 27 to 30, 1919.

*Technique for the Agglutination Tests.*—The meningococcus stock cultures were kept on serum dextrose agar or semisolid ascitic fluid agar. Transfers for agglutination tests were made on dextrose serum agar slants and incubated at 37°C. for 18 hours. Suspensions were made up with 0.85 per cent saline solution to a density equal to that of barium sulfate Standard No. 3, which approximates 2,000 million cocci per cc. The serum dilutions were made up with 0.85 per cent saline solution. The agglutination tests were set up in small racks in 3 by  $\frac{3}{8}$  inch tubes, equal parts (0.2 cc. each) of serum dilution and culture suspension being used for each tube. Incubation was at 55°C. for 24 hours. The tubes were shaken 3 hours before reading, as it had been found that shaking increased and made more uniform the agglutination titer of the serum.

In some of the tests the cultures were grown on plain dextrose agar for 18 hours at 37°C., the growth was suspended in 0.85 per cent saline solution, the suspensions were heated at 65°C. for 30 minutes, and 0.5 per cent phenol was added. They were then diluted to the required density.

*Technique for the Absorption Tests.*—For the absorption tests a method, based largely upon the observations of Tulloch<sup>1</sup> and of Gordon,<sup>2</sup> was devised and tested by suspending in a serum its homologous culture under different conditions. Two densities of culture suspension, both heated and unheated, and two temperatures of incubation were used. The results are shown in Table I. Satisfactory absorption of the agglutinins took place only when the heated, dense suspensions of culture, incubated at 55°C., were used.

A dense suspension containing about 30,000 million cocci per cc. was secured by suspending in 0.85 per cent saline solution the 18 hour growth on dextrose agar, which had been incubated at 37°C., then heating to 65°C. for  $\frac{1}{2}$  hour, and adding 0.5 per cent phenol.

<sup>1</sup> Tulloch, W. J., On the differentiation by means of the absorption of agglutinin test for types of meningococci (etc.), *J. Roy. Army Med. Corps*, 1917, xxix, 66.

<sup>2</sup> Gordon, M. H., Bacteriological studies in the pathology and preventive control of cerebro-spinal fever among the forces during 1915 and 1916. I.—The definition of the meningococcus, *Med. Research Com., Nat. Health Insurance, Special Rep. Series*, No. 3, 1917, 10.



Equal parts of the serum, diluted to 1:25 with saline solution, and the suspension of cocci to be tested were thoroughly mixed and incubated at 55°C. for 2 hours and then placed in the ice box at 5–8°C. The next morning the tubes were centrifugalized at high speed for  $\frac{1}{2}$  hour, or longer if necessary, to throw down all the bacteria, and the superna-

TABLE I.

*Degrees of Absorption with Heated and Unheated Suspension, Incubated at 37° and 55°C., and a Thin and a Dense Suspension.*

A Type II serum was saturated with its homologous culture under varying conditions, and after incubation and centrifugalization the supernatant fluid was decanted and tested in a series of dilutions against its homologous culture according to the standard method.

Suspension.	No. of cocci per cc.	Temperature at which culture was heated for $\frac{1}{2}$ hr.	Temperature at which culture was incubated with serum for 2 hrs.	Agglutination of supernatant fluid with homologous culture, Type II.
	<i>millions</i>	°C.	°C.	
A	5,000	65	55	1:400
AA	5,000	65	37	1:400
B	30,000	65	55	0
BB	30,000	65	37	1:100
C	5,000		55	1:600
CC	5,000		37	1:600
D	30,000		55	1:300
DD	30,000		37	1:600

tant fluid was decanted and tested against the homologous culture of the serum. A sample of the same serum was incubated, centrifugalized, and tested in the same dilutions as the saturated serum.

The monovalent type serums were produced in rabbits according to the method suggested by Amoss.<sup>3</sup>

#### RESULTS.

Standard suspensions and serums of the four types of Gordon were sent to this laboratory by the British Medical Research Committee. Living cultures of the four types of meningococci were sent

<sup>3</sup> Amoss, H. L., quoted by Flexner, S., Mode of infection, means of prevention and specific treatment of epidemic meningitis, *J. Am. Med. Assn.*, 1917, lxix, 639.

to us by Dr. W. H. Park, of the Department of Health of the City of New York, who had received them from Colonel Gordon. Tests were made of the standard suspensions with the standard serums and with our monovalent normal, para, and irregular serums; also the standard serums were tested with our stock cultures. The results of the tests were not altogether clear-cut and satisfactory because the standard serums and suspensions were not tested until after the expiration date recorded on them. It was very clearly indicated, however, that Types I, II, and III of Gordon corresponded respectively to the normal, para, and irregular types. The same relation was indicated by a study of the living cultures of Types I, II, and III of Gordon.

We were unable to designate any of the cultures of our series as belonging to Type IV of Gordon. The Type IV standard suspension showed only a trace of agglutination in the homologous serum, but when tested with the normal, para, and irregular serums, it was agglutinated in a 1:100 dilution by the para type serum, and in a 1:300 dilution by the irregular type serum. These reactions, together with those of the Type IV living culture from Dr. Park, seem to indicate that Type IV may not be a separate group but possibly a poorly agglutinating strain belonging to Type II or III.

Agglutination tests were made with 53 stock cultures in the monovalent rabbit serums produced against the selected strains of normal, para, and irregular meningococci. 43 of the 53 cultures were classified into three distinct groups: six as Type I, thirteen as Type II, and twenty-four as Type III. One culture was agglutinated in Serums I and III, two groups which are said to be related. Nine cultures could not be classified into these groups because they did not agglutinate in sufficiently high dilutions of the four monovalent type serums which were used to classify the other cultures. The type serums employed comprised one of Type I, two of Type II, and one of Type III. Some of the unclassified cultures might conceivably be cultures which were not readily agglutinated. Others might only have failed to react with the particular monovalent serum with which they were tested. With monovalent serums produced with other representatives of the groups they might have been agglutinated.

The three types, Nos. I, II, and III, the recognition of which was based upon the agglutination test, seemed to be distinct when the absorption test was used, in that Type I culture absorbed the specific agglutinins from Type I serum, and did not absorb them from Type II or Type III serum; likewise Type II and Type III cultures absorbed the agglutinins from their respective homologous serums and not from the other types.

*Stability of the Serological Types of Meningococci.*

The constancy of the reactions of meningococcus strains has at times been questioned; that is, whether a culture at one time gives reactions that would indicate its classification in one group and at a later time its classification in another group. The reactions with at least some strains may be constant for considerable periods of time, for tests at weekly intervals with six strains under cultivation on the standard media used for all strains for 2 years have shown no change so far as the agglutination reaction is concerned.

*Variations in Reaction of Four Type II Cultures and One Type IV.*

A study was made of the variations in reaction among the cultures of our series. We record here the differences found in some of the Type II meningococcus cultures, and in the culture which we received as a Gordon Type IV.

Tulloch<sup>4</sup> and others have noted that the strains of Type II differ in their agglutination reactions, and Dopter and Pauron<sup>5</sup> suggest that their group of parameningococci which corresponds to the Type II of Gordon might be divided into three subgroups. Both French and British workers have noted the relation between Types II and IV of Gordon.

Monovalent serums obtained from rabbits immunized against Type II Cultures 1 and 4 agglutinated Type II Cultures 1, 4, 46, and G2 (the Gordon Type II culture received from Dr. Park). Monovalent

<sup>4</sup> Tulloch, W. J., Agglutination and absorption of agglutinin reaction, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

<sup>5</sup> Dopter and Pauron, Différenciation des paraméningocoques entre eux par la saturation des agglutinins, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

serums obtained by immunization with Type II Cultures 46 and G2 agglutinated in high dilution Type II Cultures 46 and G2, but did not agglutinate Type II Cultures 1 and 4 except in low dilution.

Type II Cultures 46 and G2 absorbed the agglutinins from serum obtained by immunization with Type II Culture 4, and these cultures also absorbed the Type II agglutinins from serum obtained by immunization with Cultures 46 and G2. It is evident that the agglutination reaction alone failed to bring out the relation of these cultures, but that in absorption tests they reacted similarly. It is nevertheless evident from the agglutination reaction that Cultures 1 and 4 might be said to represent one subgroup and Cultures 46 and G2 another.

No cultures were secured which typified Gordon's Type IV save the culture which came originally from Gordon and which we designated as G4. A monovalent serum obtained from a rabbit immunized with this culture agglutinated in high dilution Type II Cultures 1, 4, 46, and G2, but did not agglutinate its homologous culture, No. G4, except in low dilution. It is, therefore, a culture which is not readily agglutinated but seems to be active as an antigen. Culture G4 did not absorb the agglutinins for Type II cultures from the serum of a rabbit immunized with Type II Culture 4, but absorbed these agglutinins from serums obtained by immunization with Nos. 46 and G2. Although not readily agglutinable, the culture was apparently active as an antigen in the development of Type II agglutinins, and was capable of absorbing agglutinins for some, but not all Type II cultures from the homologous serum. Although a relation between Culture G4 and the second group of Gordon was thus indicated, it was the only culture of our series which was sufficiently definite in its reactions to suggest the fourth group of Gordon.<sup>6</sup>

It seems possible that Type II is a complex group and that its members cannot be properly differentiated by the agglutination reaction, although strains which react typically may be classified in it.

These irregular reactions of agglutination and absorption suggested that the agglutination reaction is not a satisfactory basis for a final

<sup>6</sup> Further study is now being made of the variations in agglutination and absorption of the cultures which have been reported to comprise the fourth group of Gordon.

classification, although it might be of value in differentiating groups so that representative strains could be selected for certain purposes such as the immunization of animals to obtain polyvalent serums. That the selection of the representative strains based upon the results of this study was of value in producing a polyvalent serum in horses is well shown by the fact that such serum agglutinated all cultures of our series in high dilution.

#### CONCLUSION.

The meningococcus, like some other pathogenic species, varies in its agglutination in immune serum, some strains being readily agglutinable while others agglutinate with difficulty in their homologous serum as well as in heterologous serums. The different strains appear to vary also in their action as antigens. In order to secure representative strains, therefore, it was thought necessary to consider the antigenic action as well as the agglutinability of the cultures.



COMPARISON OF THE POTENCY OF POLYVALENT ANTI-MENINGOCOCCUS SERUM PRODUCED WITH FOUR AND SIX REPRESENTATIVE STRAINS AND THAT PRODUCED WITH SIXTY STRAINS, AS DETERMINED BY THE AGGLUTINATION TITER.\*

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(Received for publication, July 9, 1920.)

In the treatment of cerebrospinal fever by antimeningococcus serum the polyvalency of the serum has been considered of the utmost importance. Yet the potency of the polyvalent serum against the individual strain may be sacrificed by immunizing with a large number of strains. The technical difficulties of developing such a serum make its production onerous, while the methods of testing its therapeutic potency are also burdensome and far from satisfactory. Furthermore, in New York State the regulations for the production and standardization of antimeningococcus serum for sale require immunization with and tests against only four representative strains. Manufacturers then might not use more than the four strains required and it is important to determine what effect this procedure would have on the polyvalency of the serum.

Although the agglutination test is not a satisfactory method of determining therapeutic potency, it is the only one that suggests the degree of polyvalency. The practical value of complement fixation or of measuring the bacteriotropin content has not been demonstrated, and there is no satisfactory protection test.

In this study one horse was immunized with four strains, another with six, and the serums obtained from these horses were compared

\* Presented at the 47th annual meeting of the American Public Health Association, New Orleans, La., October 27 to 30, 1919.

with the serum obtained from a horse which had been immunized with the regular stock meningococcus cultures—60 strains in all, including the six which were used in the immunization of the horses under investigation. The serum from the horse immunized with the large number of cultures was representative of the classical method, since it had been found to compare favorably with serum produced by The Rockefeller Institute and the Research Laboratory of the Department of Health of the City of New York.

The problem of selecting representative strains was obviously difficult on account of the obstacles encountered in the classification of the meningococci which have been obtained from cases of cerebro-spinal meningitis, from carriers, and from healthy persons. The four representative or standard strains selected included one regular strain, one parameningococcus, and two intermediate strains. One horse was immunized with these four cultures; the other horse was immunized with two cultures of each of the three main groups. These strains represented the groups recognized in the classifications of Dopter,<sup>1</sup> Gordon,<sup>2</sup> Flexner,<sup>3</sup> and others. When only four or six representative strains were used the horse received at each dose an equal quantity of each strain. The dose was increased and adjusted to give rise to approximately the same degree of reaction that was obtained in the immunization with a large number of strains.

### *Method.*

The method of preparing the cultures and immunizing the horses with suspensions of them was similar whether 4, 6, or 60 strains were used for immunization. The cultures were grown on serum dextrose agar slants for 17 to 19 hours at 37°C. and were suspended in 0.85 per cent salt solution. The inoculations were made intravenously

<sup>1</sup> Dopter, C., Étude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques), *Compt. rend. Soc. biol.*, 1909, lxxvii, 74.

<sup>2</sup> Gordon, M. H., Bacteriological studies in the pathology and preventive control of cerebro-spinal fever among the forces during 1915 and 1916. II.—The definition of the meningococcus, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

<sup>3</sup> Flexner, S., Mode of infection, means of prevention and specific treatment of epidemic meningitis, *J. Am. Med. Assn.*, 1917, lxi, 639.



without delay. At the beginning of immunization the first dose was a fraction of a single culture. When a large number of strains was used new strains were added as fast as possible, until twenty were injected daily. Two inoculations were made on each day, 1 hour apart, with ten cultures for each inoculation. This was done to decrease the severity of the reaction. The doses were given on 3 successive days followed by a week of rest, after which another series was begun. The cultures and the method of injection were chosen so that the whole series of stock cultures was injected as often as possible. The increase of dosage was dependent upon the temperature reaction after inoculation, the aim being to secure a rise in temperature to 104° or 105°F. 5 hours after inoculation, followed by a fall to normal the next morning.

Trial or complete bleedings were made on the 6th day after every other series of injections. The immunization of the two horses with the four and six representative strains was begun in May, 1918, and was continued for a period of more than 1 year. The largest dose was a total of 60 cc. of suspension, representing the growth from ten slants of culture. The horses were allowed to rest for 2 months, during August and September, 1918. The potency of the serum was tested according to the standard methods described in a previous paper.<sup>4</sup>

Cultures were grown at 37°C. for 18 hours on serum dextrose agar and then suspended in 0.85 per cent salt solution and diluted to a density equal to that of barium sulfate Standard No. 3, or approximately 2,000 million cocci per cc. In all the tests saline solution, normal horse serum, and type serum controls were used, as well as the standard antimeningococcus serum adopted by this laboratory. Although the standard serum has been used during a period of 2 years, it has shown practically no deterioration in agglutination titer.

According to these standard methods the agglutination titer was determined by the maximum dilution in which definite but not necessarily complete agglutination occurred. Reading the limit of definite

<sup>4</sup> Wadsworth, A. B., Kirkbride, M. B., and Gilbert, R., The standardization of antimeningococcus and antipneumococcus serums, *Arch. Int. Med.*, 1919, xxiii, 269.

agglutination, rather than of complete agglutination, has been found to be more accurate and practical. Complete agglutination occurs in a much lower dilution, and if a sufficient number of dilutions of serum are used to grade the degree of reaction accurately, different workers may experience difficulty in reading the complete agglutination. Furthermore, the ability of certain cultures to be agglutinated completely, and that of certain serums to agglutinate, vary. Finally, the percentage of discrepancy between the readings of different workers when the upper limits of agglutination are read and high dilutions are made of the serum is not great, especially if the dilutions which are doubtful are always considered negative, and only the highest dilution which shows definite agglutination is read.

#### RESULTS.

In Tables I and II are recorded agglutination tests on the serums of the two horses during immunization with four and six strains respectively in comparison with the serum of a horse immunized with 60 strains during a period of 18 months (Table III). The tests were made with the six cultures used in the immunization of the horse mentioned above. The rise in agglutination titer is very rapid during the first few weeks of immunization. Then it is slower until a constantly high titer has been reached, which, however, varies at times quite markedly, doubtless on account of a dose that was too large or too small. The potency as indicated by the agglutination titer with homologous strains was increased three- to tenfold by limiting the number of strains used in immunization.

Agglutination tests have been made with the serums of horses immunized with 4, 6, and 60 strains of meningococci against 77 homologous and heterologous cultures, including also some strains recently isolated from cases, and others reputed to belong, because of their serological reactions, to the fourth group of Gordon, typical strains of which are not commonly found in this country. From Tables IV and V it is evident that many of the heterologous type cultures were agglutinated to an even higher degree than the homologous cultures. Practically all the twelve unclassified cultures which were used in the immunization of the 60 strain horse, and also the twelve additional cultures, none of which was used in the immuni-

TABLE I.  
*Agglutination Titer of the Serum of Horse 49 during Immunization with Four Strains of Meningococci.*

Time of test.	Date.	Agglutination titer tested with different cultures.					
		No. 1B*	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B*
<i>1918</i>							
Normal serum.....	May 3	1:50	1:100	1:100	1:50	1:50	1:100
After 2nd series of injections.....	" 22	1: <200	1:1,000	1:2,000	1:5,000	1:4,000	1:5,000
" 4th " "	June 10	1:1,000	1:2,000	1:3,000	1:8,000	1:5,000	1:7,000
" 6th " "	" 26	1:2,000	1:4,000	1:3,000	1:12,000	1:6,000	1:8,000
" 8th " "	July 15	1:1,000	1:3,000	1:4,000	1:9,000	1:6,000	1:7,000
" 10th " "	Aug. 2	1:3,000	1:5,000	1:5,000	1:12,000	1:8,000	1:9,000
" 2 mos. rest.....	Oct. 14	1:1,000	1:1,000	1:2,000	1:10,000	1:2,000	1:4,000
" 12th series of injections.....	Nov. 1	1: <2,000	1:4,000	1:4,000	1:6,000	1:6,000	1:6,000
" 14th " "	" 21	1:2,000	1:4,000	1:4,000	1:8,000	1:8,000	1:10,000
" 16th " "	Dec. 11	1:2,000	1:3,000	1:4,000	1:8,000	1:3,000	1:7,000
" 18th " "	" 30	1:2,000	1:2,000	1:4,000	1:10,000	1:8,000	1:10,000
<i>1919</i>							
" 19th " "	Jan. 8	1:2,500	1:4,000	1:5,500	1:11,000	1:5,500	1:8,500
" 21st " "	Feb. 1	1:1,000	1:4,000	1:4,000	1:12,000	1:4,000	1:8,000
" 23rd " "	" 21	1:1,000	1:4,000	1:4,000	1:12,000	1:4,000	1:4,000
" 24th " "	Mar. 3	1:1,000	1:2,000	1:4,000	1:12,000	1:5,000	1:9,000
" 26th " "	" 28	1:1,500	1:2,500	1:3,500	1:11,500	1:6,000	1:8,000
" 28th " "	Apr. 21	1:3,000	1:3,000	1:4,000	1:12,000	1:5,000	1:8,000
" 30th " "	June 14	1:4,000	1:4,000	1:6,000	1:12,000	1:4,000	1:8,000
" 34th " "	" 30	1:2,000	1:3,000	1:4,500	1:12,000	1:6,500	1:6,500

\* These cultures were not used in the immunization of Horse 49.

In Tables I, II, and III the figures of dilution represent the highest dilution in which agglutination visible to the naked eye took place. < a certain dilution indicates that a lower dilution was not tested. Due to the fact that the end-points of agglutination were not determined in early tests, the tests with serums from May 3, 1918, through Aug. 2, 1918, have been repeated after 1 to 1½ years. The other tests represent the titers obtained within about 3 months after bleeding. Immunization was discontinued from Aug. 2, 1918, through Oct. 14, 1918. When two or more tests were made on the same serum, the average was recorded to the nearest 500, except in Table III.

TABLE II.

*Agglutination Titer of the Serum of Horse 40 during Immunization with Six Strains of Meningococci.*

Time of test.	Date.	Agglutination titer tested with different cultures.					
		No. 1B	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B
		1918					
Normal serum . . . . .	May 3	1: < 50	1: < 50	1: 50	1: 100	1: 50	1: 100
After 2nd series of injections . . . . .	" 22	1: 500	1: 3,000	1: 2,000	1: 1,000	1: 3,000	1: 3,000
" 4th " " . . . . .	June 10	1: 2,000	1: 4,000	1: 4,000	1: 8,000	1: 6,000	1: 8,000
" 6th " " . . . . .	" 26	1: 3,000	1: 8,000	1: 6,000	1: 12,000	1: 9,000	1: 12,000
" 8th " " . . . . .	July 15	1: 4,000	1: 5,000	1: 4,000	1: 12,000	1: 10,000	1: 10,000
" 10th " " . . . . .	Aug. 2	1: 3,000	1: 6,000	1: 5,000	1: 12,000	1: 10,000	1: 11,000
" 2 mos. rest . . . . .	Oct. 14	1: < 1,000	1: 1,000	1: 1,000	1: 8,000	1: 8,000	1: 4,000
" 12th series of injections . . . . .	Nov. 1	1: 2,000	1: 1,500	1: 3,000	1: 8,000	1: 8,000	1: 10,000
" 14th " " . . . . .	" 21	1: 2,000	1: 1,000	1: 4,000	1: 10,000	1: 4,000	1: 10,000
" 16th " " . . . . .	Dec. 11	1: 2,000	1: 4,000	1: 2,000	1: 10,000	1: 5,000	1: 8,000
" 18th " " . . . . .	" 30	1: 2,000	1: 2,000	1: 4,000	1: 12,000	1: 4,000	1: 10,000
1919							
" 19th " " . . . . .	Jan. 8	1: 2,000	1: 3,000	1: 4,500	1: 11,500	1: 7,500	1: 9,000
" 21st " " . . . . .	Feb. 1	1: 2,000	1: 4,000	1: 2,000	1: 12,000	1: 4,000	1: 8,000
" 23rd " " . . . . .	" 21	1: 2,000	1: 4,000	1: 4,000	1: 12,000	1: 8,000	1: 8,000
" 24th " " . . . . .	Mar. 3	1: 2,000	1: 2,000	1: 5,000	1: 12,000	1: 9,000	1: 8,000
" 26th " " . . . . .	" 28	1: 2,000	1: 4,000	1: 8,000	1: 12,000	1: 12,000	1: 8,000
" 28th " " . . . . .	Apr. 21	1: 3,000	1: 4,000	1: 7,000	1: 12,000	1: 7,000	1: 12,000
" 30th " " . . . . .	May 14	1: 4,000	1: 6,000	1: 6,000	1: 12,000	1: 6,000	1: 12,000
" 34th " " . . . . .	June 30	1: 2,000	1: 2,500	1: 4,500	1: 12,000	1: 6,500	1: 6,500

zation of any of the horses, were agglutinated in the serum of Horses 40 and 49 in higher dilution than in that of the 60 strain horse. The most radical difference in the action of the serums of Horses 40 and 49 is with Culture 286, which agglutinated in 1:600 dilution with the serum of Horse 50 (60 strains), in 1:2,000 with the serum of Horse 40, and in 1:200 with the serum of Horse 49.

The serums obtained from horses immunized with four and with six representative strains as compared with the serum of a horse immunized with 60 stock meningococcus cultures thus possessed a higher agglutination titer. This was marked when the serum was tested against the homologous strains; but a high titer was also found when the serums were tested against all the heterologous strains. Evidently, by the use of too large a number of strains the potency of the serum, as judged from the agglutination titer, is sacrificed.

TABLE III.

*Agglutination Titer of the Serum of Horse 50 Immunized with 60 Strains of Meningococci for 18 Months.*

Date.	Agglutination titer tested with different cultures.					
	No. 1B	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B
1917						
Nov. 14.....	1:700	1:800	1:1,000	1:1,100	1:900	1:1,100

The titer of a polyvalent serum obtained by immunization with 60 strains for 18 months approximated dilutions of 1:700, 1:800, 1:1,000, 1:1,100, 1:900, and 1:1,100 respectively with six representative strains of the different types of meningococci, whereas the horse immunized with these six strains possessed an agglutination titer of 1:4,000, 1:6,000, 1:6,000, 1:12,000, 1:6,000, and 1:12,000 respectively. The horse immunized with only four of the strains possessed an agglutination titer of 1:4,000, 1:4,000, 1:6,000, 1:12,000, 1:4,000, and 1:8,000 respectively, the first and last reactions being heterologous and the others homologous; that is, tests with the cultures used in immunization of the horse. There is thus marked variation in the titer obtained with the different strains, largely owing to the antigenic properties or agglutinability of the cultures. The titer apparently fluctuated to some extent while the horses were in a high

TABLE IV.  
Results of Agglutination Tests of *Meningococcus* Cultures with Experimental Polyvalent Antimeningococcus Serums.

Culture No.	Classification of cultures.		Complete agglutination.*				Maximum agglutination.†			
			Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.		Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.	
	Gordon nomenclature.	Ordinary nomenclature.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.
1	Type II	Para.	— ‡	1:1,000	1:1,000	1:1,000	—	1:2,000	1:4,000	1:4,000
2	" III	Irregular.	—	1:1,000	1:1,000	1:1,000	1:500	1:4,000	1:4,000	1:8,000
3	" III	"	—	1:1,000	1:500	1:1,000	1:500	1:4,000	1:2,000	1:4,000
4B	" II	Para.	—	1:1,000	1:1,000	1:1,000	—	1:8,000	1:8,000	1:8,000
5	Unclassified.	Unclassified.	—	—	—	—	—	—	—	—
6	Type II	Para.	—	1:1,000	1:500	1:1,000	1:500	1:4,000	1:4,000	1:4,000
7	" III	Irregular.	—	1:1,000	1:500	1:1,000	1:500	1:2,000	1:2,000	1:2,000
8	" II	Para.	—	—	—	—	1:1,000	1:4,000	1:4,000	1:2,000
9	Unclassified.	Unclassified.	—	—	—	—	—	—	—	1:500
10	Type III	Irregular.	—	—	1:500	1:500	1:1,000	1:4,000	1:4,000	1:8,000
30	" III	"	—	1:1,000	1:1,000	1:2,000	1:1,000	1:8,000	1:12,000	1:8,000
31	" III	"	—	1:500	1:500	1:1,000	1:500	1:2,000	1:8,000	1:12,000
32	Unclassified.	Unclassified.	—	1:1,000	1:1,000	1:1,000	1:1,000	1:4,000	1:4,000	1:4,000
33	Type II	Para.	—	1:500	1:500	1:500	1:1,000	1:1,000	1:1,000	1:1,000
35A	" II	"	—	1:500	1:500	1:500	1:1,000	1:1,000	1:1,000	1:500
36	" III	Irregular.	—	1:8,000	1:8,000	1:8,000	1:2,000	1:12,000	1:12,000	1:12,000
37	" III	"	—	1:1,000	1:1,000	1:1,000	1:2,000	1:8,000	1:4,000	1:2,000
38A	Types I and III	{ Normal. " }	—	1:1,000	1:1,000	1:500	—	1:8,000	1:8,000	1:8,000
39	Type I	"	1:500	1:4,000	1:4,000	1:2,000	1:1,000	1:12,000	1:12,000	1:12,000

40	Type II	Para.	—	—	—	—	1:1,000	1:2,000	1:1,000	1:4,000	1:4,000	1:1,000
42	" III	Irregular.	—	—	—	—	1:1,000	1:2,000	1:1,000	1:8,000	1:4,000	1:4,000
43	" II	Para.	—	—	—	1:500	—	—	—	1:2,000	1:4,000	1:2,000
44	" II	"	—	—	—	1:500	1:500	—	—	1:1,000	1:500	1:1,000
45	" III	Irregular.	—	—	—	1:500	1:500	1:500	1:500	1:1,000	1:4,000	1:4,000
46	" II	Para.	—	—	—	1:4,000	1:4,000	1:4,000	1:8,000	1:12,000	1:8,000	1:12,000
48	" III	Irregular.	—	—	—	1:500	1:1,000	1:1,000	1:500	1:4,000	1:8,000	1:4,000
49	" III	"	—	—	—	—	—	—	—	1:4,000	1:2,000	1:4,000
50	" III	"	—	—	—	1:2,000	1:2,000	1:4,000	1:1,000	1:500	1:1,000	1:2,000
60	" I	Normal.	—	—	—	1:4,000	1:2,000	1:1,000	1:1,000	1:12,000	1:12,000	1:12,000
61	Unclassified.	Unclassified.	—	—	—	1:4,000	1:2,000	1:1,000	1:8,000	1:12,000	1:4,000	1:12,000
62	Type II	Para.	—	—	—	—	—	—	—	1:1,000	1:500	1:1,000
63	" I	Normal.	1:500	1:2,000	1:2,000	1:2,000	1:1,000	1:1,000	1:1,000	1:4,000	1:4,000	1:4,000
64	" III	Irregular.	—	—	—	1:500	1:500	1:1,000	1:500	1:2,000	1:4,000	1:4,000
65	Unclassified.	Unclassified.	—	—	—	—	—	—	—	1:1,000	1:1,000	1:1,000
66	Type III	Irregular.	—	—	—	1:500	1:500	1:2,000	1:1,000	1:12,000	1:12,000	1:12,000
67	" III	"	—	—	—	—	—	—	—	1:500	1:500	1:500
68B	" III	"	—	—	—	—	—	—	—	1:500	—	—
69	" III	"	—	—	—	1:1,000	1:1,000	1:1,000	1:1,000	1:2,000	1:4,000	1:2,000
70	Unclassified.	Unclassified.	—	—	—	1:1,000	1:1,000	1:1,000	1:500	1:4,000	1:4,000	1:2,000
71	Type III	Irregular.	—	—	—	1:500	1:500	1:500	—	1:2,000	1:4,000	1:2,000
72	" II	Para.	—	—	—	1:500	1:1,000	1:500	—	1:500	1:2,000	1:1,000
73	Unclassified.	Unclassified.	—	—	—	—	—	—	—	1:1,000	1:500	1:1,000
74	Type III	Irregular.	—	—	—	1:500	1:1,000	1:500	—	1:500	1:2,000	1:2,000
75	" III	"	—	—	—	1:1,000	1:1,000	1:1,000	1:1,000	1:8,000	1:12,000	1:8,000
76	" I	Normal.	—	—	—	1:1,000	1:1,000	1:1,000	1:500	1:12,000	1:4,000	1:4,000
77	" I	"	—	—	—	1:1,000	1:2,000	1:500	1:1,000	1:4,000	1:4,000	1:2,000
78	" III	Irregular.	—	—	—	1:1,000	1:1,000	1:1,000	1:1,000	1:4,000	1:8,000	1:12,000
79	" I	Normal.	—	—	—	1:1,000	1:2,000	1:1,000	1:1,000	1:12,000	1:8,000	1:4,000
80	" III	Irregular.	—	—	—	—	1:500	1:500	1:500	1:4,000	1:1,000	1:1,000

\* The dilution in which the supernatant fluid is clear.

† The standard maximum agglutination titer.

‡ Blank indicates that the culture was not tested; —, that the reaction was not obtained in the lowest dilution tested, 1:500, except as otherwise indicated; namely, 1:200 with the New York Hospital culture and No. 55, and with the cultures in Table V.

TABLE IV—*Concluded.*

Culture No.	Classification of cultures.		Complete agglutination.*						Maximum agglutination.†						
			Serum of Horse 40 immunized with 6 strains of meningococci.			Serum of Horse 49 immunized with 4 strains of meningococci.			Serum of Horse 50 immunized with 60 strains of meningococci.		Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.		
			Serum of Horse 50 immunized with 60 strains of meningococci.	July 15, 1918.	Dec. 30, 1918.	Serum of Horse 40 immunized with 6 strains of meningococci.	Aug. 2, 1918.	Dec. 30, 1918.	Serum of Horse 49 immunized with 4 strains of meningococci.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Dec. 30, 1918.	
81	Ordinary nomenclature.	Gordon nomenclature.	Nov. 14, 1917.	—	—	—	—	—	1:500	1:1,000	1:2,000	1:1,000	1:1,000		
82				—	—	1:500	1:1,000	—	1:500	1:4,000	1:2,000	1:500	1:4,000	1:500	
84				—	—	—	—	—	—	—	1:1,000	1:500	1:1,000	1:2,000	
1B				—	—	—	—	—	—	—	1:2,000	1:2,000	1:2,000	1:2,000	
10B				—	1:500	1:500	1:500	1:500	1:500	1:1,000	1:2,000	1:2,000	1:2,000	1:2,000	
W30B				—	—	1:1,000	1:1,000	1:1,000	1:2,000	1:2,000	1:2,000	1:8,000	1:8,000	1:8,000	
46B				—	1:2,000	1:4,000	1:2,000	1:4,000	1:2,000	1:12,000	1:12,000	1:12,000	1:12,000	1:12,000	
W60B				—	1:2,000	1:2,000	1:2,000	1:1,000	1:500	1:8,000	1:8,000	1:8,000	1:8,000	1:4,000	
79B				—	1:2,000	1:2,000	1:2,000	1:1,000	1:2,000	1:8,000	1:12,000	1:12,000	1:12,000	1:8,000	
Type II (Gordon).				—	1:2,000	1:1,000	1:1,000	1:500	—	1:12,000	1:12,000	1:12,000	1:12,000	1:12,000	
Type IV (Gordon).	?	IV	—	—	1:500	1:1,000	1:1,000	1:500	—	1:2,000	1:2,000	1:2,000	1:2,000		
105				—	—	—	—	—	—	—	—	—	—	—	
New York Hospital. 55				—	Mar. 28, 1919.	Mar. 28, 1919.	Mar. 28, 1919.	—	—	—	—	—	—	—	—
				—	—	—	—	—	—	—	—	—	—	—	—
				—	June 30, 1919.	June 30, 1919.	June 30, 1919.	—	—	—	—	—	—	—	—
				—	—	—	—	—	—	—	—	—	—	—	—
				—	1:2,000	—	—	—	—	—	—	—	—	—	—



TABLE V.  
*Results Obtained with Cultures Received from Dr. McCoy.\**

Culture No.	Classification of cultures.			Complete agglutination:†				Maximum agglutination:‡			
	Gordon nomenclature.	Ordinary nomenclature.	McCoy's classification.	Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.	Serum of Horse 49 immunized with 4 strains of meningococci.	Serum of Horse 49 immunized with 6 strains of meningococci.	Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.	Serum of Horse 49 immunized with 4 strains of meningococci.	Serum of Horse 49 immunized with 6 strains of meningococci.
11	Type I	Normal.	Type I	Nov. 14, 1917.	Mar. 28, 1919.	Mar. 28, 1919.	Nov. 14, 1917.	Mar. 28, 1919.	Mar. 28, 1919.	Mar. 28, 1919.	Mar. 28, 1919.
123	" I	"	" I	—	1:500	1:200	1:800	1:5,000	1:5,000	1:5,000	1:5,000
128	" III	Irregular.	Not given.	—	1:4,000	1:2,000	1:1,200	1:9,000	1:9,000	1:7,000	1:7,000
57	" III	"	Type III	—	1:800	1:2,000	1:1,000	1:10,000	1:10,000	1:12,000	1:12,000
286	Unclassified.	"	" II	—	—	1:500	1:1,200	1:3,000	1:3,000	1:3,000	1:3,000
56	Type II	Para.	" II	—	—	—	1:600	1:2,000	1:2,000	1:200	1:200
138	" III	Irregular.	" IV	—	—	—	1:800	1:4,000	1:4,000	1:3,000	1:3,000
289	Unclassified.	"	" IV	—	1:1,000	1:2,000	1:1,000	1:6,000	1:6,000	1:9,000	1:9,000
301	Type II	Para.	Indefinite.	—	—	—	1:<200	1:200	1:200	1:200	1:200
98	" III	Irregular.	Type II	—	—	—	1:800	1:2,000	1:2,000	1:3,000	1:3,000
60	Unclassified.	"	" I	—	1:800	1:800	1:1,200	1:7,000	1:7,000	1:5,000	1:5,000
307	Type III	Irregular.	" IV	—	—	—	1:200	1:800	1:800	1:800	1:800
136	" II	Para.	Not given.	1:200	1:1,000	1:800	1:1,200	1:7,000	1:7,000	1:12,000	1:12,000
			Type II	—	1:800	1:800	1:1,200	1:7,000	1:7,000	1:12,000	1:12,000
55	Type II	Para.	Type II	—	Nov. 8, 1919.	Oct. 31, 1919.	—	Nov. 8, 1919.	Nov. 8, 1919.	Oct. 31, 1919.	Oct. 31, 1919.
				—	1:800	—	1:800	1:5,000	1:5,000	1:4,000	1:4,000

\* These cultures were obtained from Dr. G. W. McCoy, of the Hygienic Laboratory, U. S. P. H.

† The dilution in which the supernatant fluid is clear.

‡ The standard maximum agglutination titer.

state of immunization, but the minimum titer of the serum from the two horses during this stage greatly exceeded the titer of the polyvalent serum from the horse immunized with 60 strains.

Since with a small number of strains, such as would meet the minimum requirements of the standards of potency for the manufacture of antimeningococcus serum in New York State, the agglutination titer may be greatly increased, as compared with previous methods in which a large number of strains was used, the minimum standards of potency should be correspondingly increased. Furthermore, the minimum standards of potency, as determined by the agglutination titer, must be adjusted for each standard culture. Until we have had more experience with the variations in titer of serums from a larger number of horses, it is, of course, difficult to determine practically how much to adjust the standard above or below the minimum agglutination titer obtained during the height of immunization in this investigation.

#### CONCLUSIONS.

The potency of a polyvalent antimeningococcus serum, as tested by its agglutination titer, was sacrificed by immunization with a large number of strains of the meningococcus. By immunization with a limited number of representative strains, four or six, carefully selected on account of their antigenic and agglutination properties, the potency was increased three- to tenfold without sacrificing the polyvalency; that is, as tested with at least 70 heterologous strains of the meningococcus.

The agglutination titer, unfortunately, is not an entirely satisfactory criterion of therapeutic potency, but it is the only practical method available that determines also the polyvalent action of antimeningococcus serum.

# PURIFICATION AND CONCENTRATION OF ANTIGENS FOR COMPLEMENT FIXATION BY METHODS OF DIALYSIS, ADSORPTION, AND EXTRACTION.\*†

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(Received for publication, July 12, 1920.)

The complement fixation test of Bordet and Gengou with bacterial antigens on the serum of highly immunized animals has been of value in the identification of bacterial species, but it has never been a practical aid in the diagnosis or prognosis of bacterial infection in man. Although the antigens which have been prepared from syphilitic tissue and even from certain normal tissues have proved to be of great value in the diagnosis of syphilis, none of the antigens which have been prepared from pure cultures of the spirochete isolated from syphilitic tissue has been reliable. On the contrary, they apparently lack reactive capacities that are characteristic for this disease. Thus the nature of the complement fixation reaction of Wassermann is obscure. The so called anticomplementary or non-specific properties of antigens which have been prepared from pure cultures of the incitants of the bacterial infection have made it difficult or impossible to standardize the test so that the characteristic reactions with the blood serum from cases of infection could be detected or studied to determine their diagnostic or prognostic value.

Prior to his work on syphilis Wassermann studied the complement fixation reaction in tuberculosis, but failed to prepare satisfactory antigens from tubercle bacilli. Recently a number of investigators have reported diagnostic reactions in so many cases of tuberculosis that there can be no question that it is possible to prepare antigens

\* Presented at the 35th annual meeting of the Association of American Physicians, Atlantic City, N. J., May 4, 1920.

† An abstract of this paper will appear in the *Proc. Soc. Exp. Biol. and Med.*

from the tubercle bacillus, and in certain stages of the disease the blood serum gives positive reactions with them; but it has not been possible to standardize the test so that it is reliable in the diagnosis of the disease or in the study of the development of immune reactions in different stages of the disease.

Work was begun in 1917, with the view of so standardizing the preparation of the antigens that it would be possible, first, to study the development of immune reactions in experimental tuberculosis in animals and, later, to determine the diagnostic value of the test in human cases of the disease. New methods of dialysis and adsorption were used in the preparation of the antigens by which the antigenic substances were greatly purified and concentrated. The purpose of this preliminary paper is to record the new methods.

#### *Preparation of Standard Immune Serums.*

All the cultures were made in a beef infusion broth containing 1 per cent of Fairchild's peptone, 0.5 per cent salt, 0.5 per cent glycerol, and titrated to react 0.5 per cent acid to phenolphthalein. The medium was sterilized fractionally in the Arnold sterilizer. Mass cultures were grown in quart or pint Blake bottles at a temperature of 37.5°C. With some cultures the growth was taken for study as soon as the pellicle had become fully developed, and with others as soon as growth had ceased. After filtration the residue of tubercle bacilli was washed with saline solution and dried partially by suction on the filter. It was then desiccated over concentrated sulfuric acid. This material was then extracted with different substances, such as carbon disulfide, ether, chloroform, and carbon tetrachloride. The fat-free residues from these extractions and also the untreated bacilli were extracted with distilled water, salt solution, sodium hydroxide, hydrochloric acid, and glycerol. The various extracts, together with the filtrates from the cultures, were tested as antigens. This technique was applied to three different strains of tubercle bacilli, a human virulent strain, a human avirulent strain, and a strain of bovine origin. In the production of immune serums by animal inoculation tuberculous organs became available which were extracted by various means and tested for antigenic properties.

The preparation of standard immune serums with these three cultures was a prolonged investigation of experimental infection and immunization of a large number of animals.<sup>1</sup> It involved not only the testing of the inoculated animals at frequent intervals but also a study of the precipitation of the globulin fraction of serum in an attempt to concentrate the antibodies. Finally, sufficiently active immune serums were obtained from horses with all three strains of the tubercle bacillus to give complete fixation of complement with the antigens which were arbitrarily selected to start the work. A single bleeding of any of the horses gave a sufficiently large quantity of serum for the titration of the comparative value of all the antigens.

*Antigenic Properties of Different Fractions of the Culture and Methods of Purification and Concentration.*

The results of the experiments in which the different fractions of the culture were tested with the standard immune serums must also be briefly summarized. The preparation of antigens by extraction of tuberculous tissues was unsatisfactory. The culture filtrates were so anticomplementary that they could rarely be used. Of all the extracts of the tubercle bacillus which have as yet been tested in our experiments, those with glycerol and, especially, distilled water produced the most active antigens. These active antigens were selected for further study by methods of dialysis and adsorption.

Animal charcoal and globulin (horse serum) were used to adsorb the substances possessing antigenic action. When animal charcoal was added the preparations lost their antigenic activity, but it is impossible to say whether this activity was adsorbed on the charcoal or destroyed as it has been as yet impossible to recover from the animal charcoal any of the antigenic properties. When globulin was added and precipitated with carbon dioxide and the precipitate extracted with alcohol, the substances possessing antigenic action with tuberculous immune serum were adsorbed and recovered in purified and concentrated condition. The so called anticomplementary action which all the original antigens possessed could not be

<sup>1</sup> This work was successfully carried on by Miss B. Johnston and will be recorded more fully in another paper.

detected in the lowest dilutions of the alcoholic extract (1:10) which could be used. The lytic action which some of the original antigens possessed also disappeared.

The technique that was used may be briefly summarized as follows: The original antigen was dialyzed, and horse serum, one part to twenty parts of antigen, was added and the mixture allowed to stand in the incubator for  $\frac{1}{2}$  hour. The globulin was then precipitated by passing purified carbon dioxide gas free from hydrochloric acid through the mixture for  $\frac{1}{2}$  hour at 37°C. The globulin precipitate was collected, and by shaking it with alcohol the antigenic substances were extracted. The alcoholic extract was concentrated in a vacuum. All the titrations were made in quantities of one-tenth of the original Wassermann test.

In order to eliminate all the dialyzable substances which prevent the adsorption of the antigenic material by globulin, the culture filtrates were dialyzed for at least 5 days. Tests of the antigenic properties at different stages of the process revealed the fact that after dialysis the filtrate became active as an antigen, and lost its anticomplementary action. After precipitation with carbon dioxide and extraction of the globulin precipitate much more active antigens were obtained. The most active antigens, however, were those obtained by this method from the distilled water extracts of the tubercle bacillus.

The following illustrates the degree of concentration and purification. Broth filtrates which were so anticomplementary that they gave no specific fixation, after dialysis were not anticomplementary and 0.02 cc. gave one unit of antigen; that is, complete fixation with the standard quantity of immune serum—two units as determined by a previous titration with one of the crude antigens which were used to standardize the immune serums. After adsorption and concentration 0.0003 cc. gave a unit of antigen. The aqueous extract antigens were anticomplementary in doses of approximately 0.05 to 0.01 cc. The results of purification and concentration of these antigens are even more striking. Before dialysis 0.003 cc. of one of them contained one unit of antigen; after dialysis it was no longer anticomplementary but owing to the increase in volume during dialysis 0.005 cc. contained one unit of antigen. After concentration 0.00015 cc. contained one unit. Expressed in terms of antigenic units per cc. this is an increase from 333 to 6,666.

The results of preliminary experiments with antigens which are used in the diagnosis of syphilis indicate that their antigenic properties may also similarly be purified and concentrated. These new methods thus open up possibilities for more precise study of many phases of infection and immunity than has hitherto been possible.

#### SUMMARY.

Antigens were prepared from the culture filtrates of tubercle bacilli and by extraction of washed and dried organisms with organic and aqueous solvents and from tissues of organs showing tuberculous lesions.

A comparison of these preparations by means of the complement fixation test showed that the aqueous extracts were most active antigenically.

The antigenic activity of the aqueous preparations and also of the slightly active culture filtrates was increased by means of dialysis and adsorption with serum globulin followed by extraction with alcohol and concentration of the alcoholic extract *in vacuo*.





# EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

## I. TRANSMISSION EXPERIMENTS WITH NASOPHARYNGEAL WASHINGS.

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PLATES 5 TO 7.

(Received for publication, July 15, 1920.)

### INTRODUCTION.

In planning the present experiments we had in mind the possible presence in the nasopharynx of persons suffering from acute epidemic influenza of some agent the effects of which might be noted in animals. In considering the criteria of activity of this agent we thought, first, of the well known phenomenon in man of leucocytic depression, involving especially the mononuclear cells, during the acute influenzal attack, and next, of changes of a more or less pronounced but possibly transient character, arising in the lungs, which might conceivably predispose to the severe pneumonias that often accompany as a secondary or concurrent infection the influenzal attack.

Furthermore, this study was made during the course of over 1½ years in three successive periods. The first period coincided with the epidemic wave of 1918-19. During this period cases of acute uncomplicated influenza and individuals who had never been affected were studied. The second period embraced the late autumn of 1919, during which influenza did not prevail in New York in epidemic form. During this interepidemic period normal individuals were studied as controls. The third period, the winter of 1920, saw a return of the epidemic. At this time additional cases of the disease were available for investigation. By proceeding in this manner we hoped to check the results for each period against the others. As the sequel will show, we believe that we succeeded in this undertaking, with the consequence that we are enabled to present our findings with perhaps a degree of confidence not otherwise appropriate.

*Materials.*

The outstanding difficulty in the choice of materials to be employed arose from the necessity of selecting cases of undoubted acute influenza, on the one hand, and of perfectly healthy individuals, who had never suffered from the disease, on the other. In the end the second requirement was more easily fulfilled, as the circumstances of the undertaking admitted of leisurely and painstaking choice of subjects. With uncomplicated influenza, however, the individuals had to be chosen at once, since the epidemic wave of the disease is notably brief, being prolonged chiefly by secondary respiratory infections.

The criteria which were used as guides in the selection of cases of pure influenza were abrupt onset with chilliness, fever, prostration, headache, and muscular pains, especially in the back and limbs. Among the early symptoms were flush and suffusion of the face, injection of the conjunctivæ, soreness of the throat, and harsh, unproductive cough. In the early stages no physical signs were detected in the chest, gastrointestinal symptoms were inconspicuous, and disturbances referable to other internal organs were not complained of or detected by physical examination.

These symptoms, although striking, were rarely such as could be measured accurately. However, there was one sign that had a quantitative value; namely, the leucocytic picture. Uncomplicated influenza shows a pronounced leucopenia affecting the absolute number of mononuclear cells, chiefly of the lymphocytic variety.<sup>1</sup> This is persistent and even resists at times secondary infectious processes, *e.g.* pneumonia, in which leucocytosis is the rule. As will appear below, great reliance was placed on this quantitative sign in the present experimental studies.

The symptoms and effects endured for from 1 to 3 days, when convalescence, initiated by a lytic fall of temperature, set in, and recovery promptly followed.

<sup>1</sup> The term mononuclears as employed by us includes the leucocytes of the lymphocytic and large mononuclear varieties which have a single homogeneous nucleus. Any indentation of the outline of the nucleus placed the cell in the transitional class, to be counted with the polymorphonuclear cells. Of the varieties of mononuclear cells, the small cells, or lymphocytes, were especially involved in the leucopenia.

Saline washings from the nose and throat were employed. These materials were secured from eight cases of influenza within the first 36 hours of the disease, and from twelve cases at later stages, including the convalescence or the period of postinfluenzal pneumonia. In addition, fourteen individuals who had not been affected were tested during the epidemic or interepidemic period.

### *Choice of Animal.*

In earlier experiments, having in mind a filterable microorganism or virus, we employed *rhesus* monkeys. But this species of animal was found to be unsatisfactory. Monkeys are at best scarce in this country and frequently suffer from pulmonary lesions of a tubercular or other type; the experiments required animals more readily available and free from respiratory affections of any nature. The rabbit was therefore chosen.

### EXPERIMENTAL.

Full grown rabbits were used for inoculation, and no rabbit suffering from snuffles or any detectable disease was employed. All animals were subjected to preliminary blood-counting, weighing, and temperature-taking, and any showing variations beyond the average were rejected.<sup>2</sup> These observations were made on 3 to 7 successive days previous to inoculation. The blood counts were carefully controlled; separate apparatus were used for each animal, which was examined throughout the course of observation by the same technician. Particular stress was laid on the total number of leucocytes and the relative and absolute numbers of polymorphonuclear and mononuclear cells.<sup>1</sup> With regard to temperature, it should be emphasized that in these animals the temperature in itself is no indication at times of the extent or absence of pathological involvement but should be interpreted only in conjunction with other findings.

<sup>2</sup> For assistance in this and other work we acknowledge our indebtedness to Captain Frank Hornaday, Medical Corps, U. S. Army, and to the Army Laboratory Technicians Miss Mary Jardine, Miss Clara M. McKee, and Miss Anne Webb.

*Mode of Inoculation.*—The inoculations were made directly into the lungs by means of the intratracheal catheter—a method slightly modified from that employed by Lamar and Meltzer<sup>3</sup>—or by tracheotomy.<sup>4</sup>

The first method, having the advantage of rapidity of operation, consisted in the insertion intratracheally by way of the mouth of a French silk catheter, size 9. An electric otoscope set in the mouth of the animal served as a gag as well as a guide for directing the catheter. The catheter was curved at 90°  $\frac{1}{2}$  inch from the tip. In inserting one should avoid slipping over the larynx into the esophagus, thereby contaminating the catheter.

Although tracheotomy requires more time, it is the preferable procedure because contamination by mouth bacteria is avoided. An incision is made directly over the trachea, and a needle, size 19, bent at a right angle, is inserted therein.

*Materials Inoculated.*—The materials employed for inoculation consisted of (a) unfiltered nasopharyngeal washings,<sup>5</sup> (b) filtered washings, (c) lung tissue suspensions,<sup>6</sup> filtered and unfiltered, from previously inoculated rabbits, (d) similar lung tissue preserved in sterile 50 per cent glycerol, (e) bacteria and culture materials, and (f) control materials. The usual dose for a 2.5 to 3 kilo rabbit was 3 cc. of these materials.

Unfiltered nasal washings were employed in the expectation that they could be purified, or rather deprived of their ordinary bacteria by successive animal passages. It was believed that if this could be accomplished there would be a better chance of preserving and possibly of causing the multiplication of some other variety of microorgan-

<sup>3</sup> Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

<sup>4</sup> With either method light ether anesthesia was given.

<sup>5</sup> Nasopharyngeal washings were obtained as follows: The patient's mouth was rinsed with warm saline solution. Then each nasal cavity was washed with 25 to 35 cc. of saline solution, the washings being returned by way of the mouth and collected in a sterile container. The entire fluid was shaken with glass beads in a mechanical shaker for 15 minutes at high speed, or until a homogeneous mixture resulted.

<sup>6</sup> Lung tissue was prepared for inoculation as follows: The selected portion of the lungs was chopped and then ground with sterile, fine, white sand, and a proportional amount of saline solution was added in the ratio of 20 cc. of saline solution to the two entire lungs. The suspension was then centrifuged at low speed and the clearer supernatant fluid employed for inoculation.

ism, more resistant and virulent perhaps, which would give to the washings from cases of uncomplicated influenza a quality lacking in others. If was, of course, realized that not in every instance could this favorable outcome be looked for. Now and then it was to be expected that a virulent pneumococcus or streptococcus would set up a pneumonia to which the animal would succumb. But if the ordinary bacteria could be suppressed by animal passages in a few instances and something survive which produced definite changes in the tissues of the rabbits—the blood and lungs, for example—the washings from cases of influenza might thus be distinguished in their effects from the washings of another origin. In this way the operation of a pathogenic agent is to be deduced, although it might not be possible to determine certainly that this agent is the inciting microbic agent of influenza. However, if a certain correspondence in tissue and other effects can be shown to exist between the individual suffering from influenza and the rabbit inoculated with materials originally derived from influenza cases and free from all ordinary bacteria, an idea as to the probable nature of the pathogenic agent is gained which encourages further investigation along the indicated lines.

#### *Inoculation of Unfiltered Washings.*

There were inoculated into the lungs of rabbits the unfiltered nasopharyngeal secretions derived from five cases of uncomplicated influenza during the first epidemic, and three during the second, in the first 36 hours of the disease, and from eleven cases during the first epidemic and one during the second in the later stages of the affection. The following effects were induced by the materials from seven of the eight early cases, but not by any from the twelve cases examined after 36 hours.

*Clinical Effects.*—From 24 to 48 hours after inoculation fever developed, associated with the ordinary signs of indisposition in a rabbit, such as listlessness and ruffled hair, and conjunctivitis. The striking feature, however, was the definite and often marked leucopenia resulting from depression of the mononuclear cells, as shown in Text-fig. 1, *a*, *b*, and *c*. If the condition was allowed to run its natural course, these symptoms endured for about 3 days, the animal then returning to

normal. If the rabbit was killed—for in the absence of infection by ordinary bacteria none died—an unusual pathological picture was revealed.

*Pathological Effects.*—The respiratory organs were affected to the exclusion of all others. No pleuritis or exudate in the pleural cavity was evident. The lungs were voluminous as a result of edema and emphysema and had a mottled hemorrhagic appearance. The hemorrhages on the surface, beneath the pleura, were diffuse or discrete, occupying areas a few millimeters in extent or covering a large part of a lobe. In addition, minute petechiæ were seen scattered over the entire surface. On section of the lungs the cut surface revealed a hemorrhagic edema; it dripped a blood-stained, frothy fluid. The hemorrhages again were either diffuse and large, or discrete and small, in the latter instance being numerous.

On microscopic section carried through various parts of the lungs the lesions were found to consist (*a*) of hemorrhagic foci, and (*b*) of edema and emphysema. The hemorrhages varied in size in accordance with the observed macroscopic appearance, some being microscopic in nature. The edema was more extensive than the hemorrhages and involved alveoli and interalveolar strands of tissue. The alveoli contained coagulated serum or red corpuscles, mononuclear cells, and also at times polymorphonuclear cells of eosinophilic type and desquamated epithelial cells. The interalveolar strands were infiltrated with mononuclear cells and large cells the foreign nature of which was not always clear. Fibrin was sometimes present in small amounts. The bronchi, also, were at times filled with erythrocytes, exfoliated and degenerated epithelia, and leucocytes. The capillaries were distended with blood.

No ordinary bacteria were seen in impression films of the lung tissue or in sections stained by Gram's or MacCallum's<sup>7</sup> method, or in aerobic or anaerobic cultures of the tissue.

The two following protocols are presented in order to show the clinical and pathological effects, regarded as typical, which arise independently of the presence of ordinary bacteria of any demonstrable kind.

<sup>7</sup> MacCallum, W. G., The pathology of the pneumonia in the United States Army camps during the winter of 1917-18, Monograph of The Rockefeller Institute for Medical Research, No. 10, New York, 1919, 47.

## PROTOCOL 1.

*Patient 8.*—M. T., adult female. Feb. 9, 1919. Onset sudden with chills and fever. Feb. 10. Photophobia; prostration; muscular pains in the back and extremities; mild unproductive cough. Temperature 37.8°C.; pulse 80. Leucocytes 8,725, of which 1,832 were mononuclears. Physical examination showed lungs to be clear; pharynx red and congested; conjunctivæ injected. No other organs affected. Cultivation of sputum and nasopharyngeal washings yielded *Pneumococcus* Type IV; no Pfeiffer bacilli. The fever persisted for 48 hours and was followed by an uneventful recovery.

*Animal Inoculation.*—The unfiltered nasopharyngeal washings obtained 20 hours after the onset of the symptoms were inoculated intratracheally into Rabbit A.<sup>8</sup>

*First Passage. Rabbit A.*—Before injection the normal blood counts showed the following results: Feb. 8, 1919. Leucocytes 13,050, of which there were 6,525 each of polymorphonuclears and mononuclears. Feb. 10. Prior to inoculation, leucocytes 14,425, of which 7,357 were polymorphonuclears and 7,068 mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 8. Feb. 11. Conjunctivitis. Leucocytes 12,550, of which 7,781 were polymorphonuclears and 4,769 mononuclears. Temperature 39.6°C. Feb. 12. Conjunctivitis persists. Leucocytes 11,450, of which 7,786 were polymorphonuclears and 3,664 mononuclears. Temperature 39.8°C.

The development within 24 hours of the conjunctivitis, the rise in temperature with depression of the leucocytes and mononuclears, which endured for 48 hours, were indications for killing the animal. Hence on Feb. 12 the animal was killed.<sup>9</sup>

*Autopsy.*—The lung condition was such as has been described as a typical effect of the inoculation.

*Aerobic Cultures.*—Cultures on the usual media remained sterile.<sup>10</sup>

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<sup>8</sup> Usually more than one rabbit was inoculated at the same time, but in this instance only the course of the rabbit used for further transmission experiments is described.

<sup>9</sup> In all experiments the animal was killed by a sharp blow, which dislocated the upper cervical vertebrae. In this way the complicating effects of ether anesthesia on the respiratory tract were avoided. The blow should be properly directed; otherwise the chest may be struck, thus causing contusion of the lung, or the skull may be broken with consequent profuse hemorrhages and aspiration of the blood, in which event the pathological picture is obscured.

<sup>10</sup> As a routine practice aerobic blood cultures were made before the animal was killed. Also pieces of lung tissue were planted in 1 per cent dextrose broth, and in this medium plus rabbit blood. Anaerobic cultures of lung tissue will be described in another communication.

*Second Passage. Rabbit B.*—Average normal leucocyte count before inoculation 10,775, of which 5,604 were mononuclears. Feb. 12, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. Feb. 13. Leucocytes 7,600, of which 3,268 were mononuclears. Feb. 14. Killed.

*Autopsy.*—The lung appearance was typical.

*Aerobic Cultures.*—Remained sterile.

*Third Passage. Rabbit C.*—Average normal leucocyte count before inoculation 15,060, of which 7,461 were mononuclears. Feb. 14, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 15. Leucocytes 5,675, of which 2,043 were mononuclears. Feb. 16. Leucocytes 8,760, of which 3,942 were mononuclears. Killed.

*Autopsy.*—Typical lung lesions, but to a milder degree.

*Aerobic Cultures.*—No growth of ordinary bacteria obtained.

A suspension of the lung tissue from this rabbit failed, however, to produce a similar effect in the succeeding rabbit. At this point the series of transmissions was terminated.

## PROTOCOL 2.

*Patient 16.*—N. L., adult female. Mar. 28, 1919. Onset sudden with chills and fever. Mar. 29. Temperature 39°C.; muscular pains in back; prostration and weakness; epistaxis; unproductive cough; coryza and photophobia. Flushed face; injected conjunctivæ; congested pharynx; lungs, a few râles anteriorly and posteriorly on left side only. No other organs affected. Leucocytes 7,300; mononuclears 2,117. Cultivation of sputum and nasopharyngeal washings yielded *Pneumococcus* Type IV. Sputum injected into mouse yielded no Pfeiffer bacilli, only the pneumococci. On the 2nd day of illness the temperature declined, and the general condition improved. On the 3rd day a relapse occurred, but recovery began on the 4th day.

*Animal Inoculation.*—The unfiltered nasopharyngeal washings were obtained 36 hours after the onset and were inoculated into the lungs of Rabbit A.

*First Passage. Rabbit A.*—Mar. 29, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 16. Mar. 30. Conjunctivitis. Leucocytes decreased from the norm of 8,200 to 6,700, and mononuclears from 4,100 to 2,680. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—Free from growth.

*Second to Sixth Passages. Rabbits B, C, D, E, and F.*—Each rabbit was injected similarly with a suspension of the lung tissue of the immediately preceding rabbit of the series. All showed uniform clinical effects. After 24 to 48 hours there developed conjunctivitis, leucopenia, mononuclear depression, and varying temperature reactions. None died, but all were killed 48 hours after the injection, except Rabbit C which was killed after 24 hours.

*Autopsy.*—There were present varying degrees of the typical lung lesions. The lung lesions of the last (sixth) rabbit passage are shown in Figs. 1, 3, and 4.



*Aerobic Cultures.*—In each instance free from growth.

Further transmissions were not made and hence this series terminated in the sixth rabbit passage.

The two series of experiments recorded in Protocols 1 and 2 indicate that definite and consistent clinical and pathological effects were induced in two series of rabbits with materials derived from the nasopharynx of recent acute cases of influenza which were independent of the presence of ordinary aerobic bacteria.

The next series of three protocols is given in order to bring out the fact that the clinical and pathological effects regarded as typical appear even when ordinary bacteria are cultivable, and also that these bacteria are suppressible through successive inoculation while the typical effects continue to occur.

### PROTOCOL 3.

*Patient 6.*—K., adult female. Jan. 27, 1919. Onset sudden with chills and fever. Jan. 28. Pains in the muscles of back and legs; prostrated and weak; severe frontal headache; mild unproductive cough. Temperature 38°C. Physical examination showed no signs in chest; conjunctivæ injected. Cultivation of sputum and nasopharyngeal washings yielded Pfeiffer bacilli and Pneumococcus Type IV. Symptoms persisted for 3 days, followed by an uneventful recovery.

*Animal Inoculation.*—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the trachea of Rabbit A.

*First Passage. Rabbit A (Text-Fig. 1, a).*—Jan. 28, 1919. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 6. Blood count of rabbit prior to inoculation showed 15,025 leucocytes, of which 8,414 were mononuclears. Jan. 29. Purulent conjunctivitis. Leucocytes 6,600, of which 2,244 were mononuclears. Jan. 30. Leucocytes 8,025, of which 2,006 were mononuclears. Killed.

*Autopsy.*—Left lung showed gray hepatization with fibrinous pleuritis (lobar pneumonia); right lung, aside from a small area of atelectasis in upper lobe, showed edema and emphysema with large numbers of petechial hemorrhages.

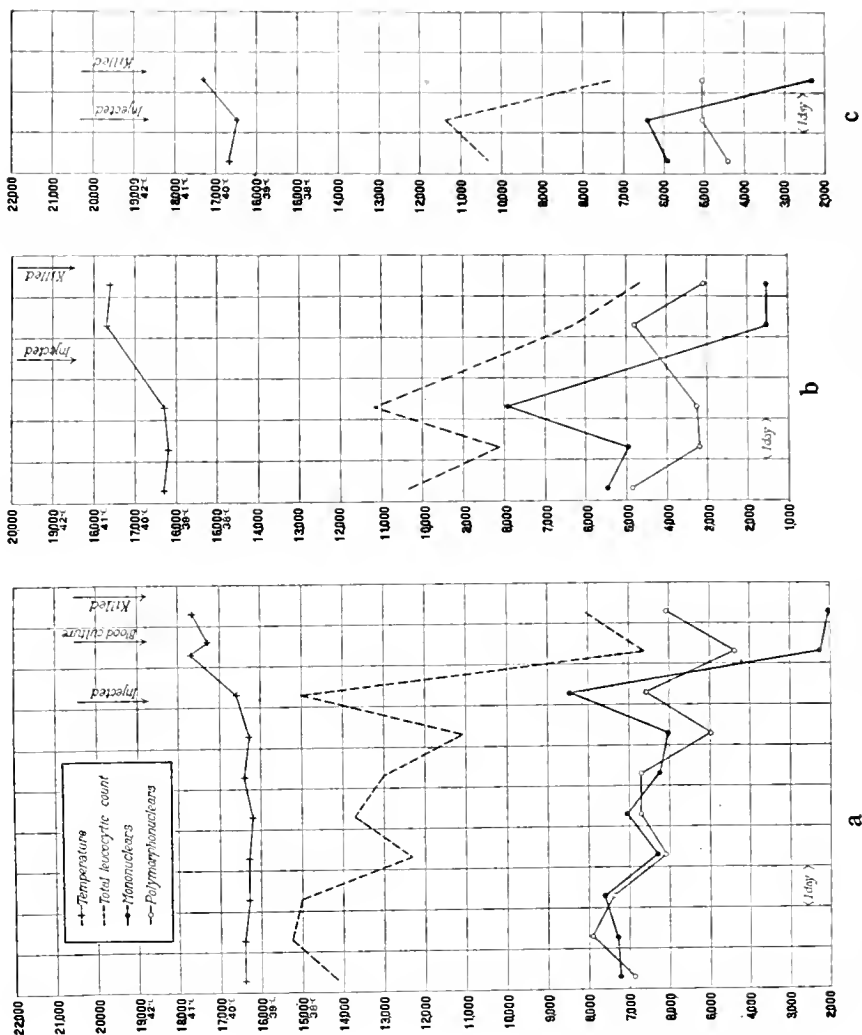
*Aerobic Cultures.*—Left lung yielded a growth of Pneumococcus Type IV, avirulent for mice;<sup>11</sup> right lung yielded no growth of ordinary bacteria.

The right lung was employed for inoculation into the next rabbit.

*Second Passage. Rabbit B (Text-Fig. 1, b).*—Jan. 30, 1919. Injected intratracheally with 3 cc. of the suspension of tissue from right lung of Rabbit A.

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<sup>11</sup> The significance of the ordinary bacteria encountered in these experiments will be dealt with in another communication.



TEXT-FIG. 1. *a*, *b*, and *c*. Effect on the blood cell count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. (*a*) First rabbit passage of the nasopharyngeal washings from a case in the early stage of uncomplicated influenza (Patient 6). (*b*) Second rabbit passage from the same case. (*c*) Seventh rabbit passage from Patient 11.

Feb. 1. Killed after having shown a depression of 6,765 in the total count and 6,269 in the mononuclear count on the 2nd day after injection.

*Autopsy.*—Both lungs were edematous and emphysematous and showed multiple punctate hemorrhages.

*Aerobic Cultures.*—No growth obtained from heart's blood or lung tissue.

*Third Passage. Rabbit C.*—Feb. 1, 1919. Normal leucocyte count before inoculation was 10,950, of which 5,585 were mononuclears. Temperature 39.1°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit B. Feb. 2. Both conjunctivæ injected. Leucocytes 9,300, of which 3,255 were mononuclears. Temperature 39.7°C. Feb. 3. Conjunctivitis. Leucocytes 7,350, of which 2,850 were mononuclears. Temperature 40.5°C. Killed.

*Autopsy.*—Lungs showed typical hemorrhagic edema and emphysema.

*Aerobic Cultures.*—No growth.

*Fourth Passage. Rabbit D.*—Feb. 3, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit C. On the next 2 days the temperature rose from 39.3° to 39.5° and 40°C. Conjunctivitis was present with a mononuclear depression of 2,455 cells on the 1st day and 1,829 on the 2nd. Feb. 5. Killed.

*Autopsy.*—Besides a few areas of consolidation at the base measuring 5 mm. in diameter, the lungs showed edema, patches of emphysema, and multiple punctate hemorrhages.

*Aerobic Cultures.*—Heart's blood, no growth; lung tissue, lower lobes *Pneumococcus* Type IV, upper lobes no growth.

The upper lobes were employed in the next transmission experiment.

*Fifth Passage. Rabbit E.*—Feb. 5, 1919. Injected intratracheally with 3 cc. of a suspension of tissue from the upper lobes of lungs of Rabbit D. During the next 2 days the temperature rose from 39.5° to 40.4° and 39.8°C. Leucopenia was noted, with a mononuclear depression from 5,377 to 2,030 on the 1st day and 3,468 on the 2nd. Feb. 7. Killed.

*Autopsy.*—Lungs showed the typical lesions similar to those of Rabbit B.

*Aerobic Cultures.*—No growth.

*Sixth Passage. Rabbit F.*—The effects were an exact repetition of those of Rabbit E so that it is unnecessary to give them in detail.

*Aerobic Cultures.*—No growth.

*Seventh Passage. Rabbit G.*—Feb. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit F. On the 2 following days the temperature rose from 39.6° to 39.8°C. Leucopenia developed, the count diminishing from 15,640 to 12,075 and 10,725 respectively, with a mononuclear depression from 8,289 to 2,415 and 3,325 cells. Conjunctivitis. Feb. 11. Killed.

*Autopsy.*—Lungs showed typical lesions similar to those of Rabbits B, C, E, and F.

*Aerobic Cultures.*—No growth.

*Eighth Passage. Rabbit H.*—Feb. 11, 1919. Temperature 39.7°C. Leucocytes 12,560, of which 5,275 were mononuclears. Injected intratracheally with

3 cc. of the suspension of lung tissue from Rabbit G. Feb. 12. Leucocytes 3,250, of which 2,015 were mononuclears. Temperature 40.35°C. Feb. 13. Leucocytes 4,300, of which 2,193 were mononuclears. Temperature 40.6°C. Died.

*Autopsy.*—Right lung showed consolidation and fibrinous pleuritis; left lung revealed typical hemorrhagic edema with emphysema.

*Aerobic Cultures.*—Left lung, no growth; right lung, *Micrococcus catarrhalis*.

The left lung was employed in the next transmission experiment.

*Ninth Passage. Rabbit I.*—Feb. 13, 1919. Leucocytes 11,200, of which 5,488 were mononuclears. Temperature 39.2°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. On the next 2 days the temperature rose to 39.6° and 39.8°C., the leucocytes diminished to 8,900 and 8,675 cells, and the mononuclears to 3,382 and 2,776 respectively. Feb. 15. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—No growth.

*Tenth Passage. Rabbit J.*—Feb. 15, 1919. Injected with lung tissue from Rabbit I with similar results.

*Eleventh Passage. Rabbit K.*—Feb. 17, 1919. Injected similarly with lung tissue from Rabbit J. For 3 days the total leucocyte count was diminished from 10,120 to 8,320, 7,125, and 7,975, and the mononuclear count from 4,250 to 2,573, 2,494, and 1,674 respectively. Allowed to recover for experiment on immunity.

This series, therefore, was voluntarily terminated in the eleventh rabbit passage. It seems certain that it could have been continued for some time and possibly indefinitely. Besides what were regarded as typical results, this series included pneumonic infections with *Pneumococcus* Type IV and *Micrococcus catarrhalis* in the first, fourth, and eighth passages. Fortunately the pneumonic areas were restricted and did not lead to infection of both lungs. Hence the successive inoculation of the bacteria-free lung tissue could be continued. The death of Rabbit H of the eighth passage was the first to occur in our experiments.

#### PROTOCOL 4.

*Patient 11.*—R. N., adult male. Feb. 18, 1919. Onset sudden with chills and fever. Feb. 19. Temperature 39°C.; pulse 80. Prostration; general malaise; coryza and photophobia; unproductive cough. Physical examination showed conjunctivitis and congested pharynx; right lung, no signs; left lung, at base, few indefinite râles. Leucocytes 5,040, of which 1,764 were mononuclears. Cultures from sputum and nasopharyngeal washings yielded only *Streptococcus viridans*. Symptoms endured for 3 days, followed by an uneventful recovery.

*Animal Inoculation.*—The unfiltered nasopharyngeal washings obtained 24 hours after the onset of the symptoms were inoculated into the lungs of Rabbit A.

*First Passage. Rabbit A.*—Feb. 19, 1919. Leucocytes 13,960, of which 8,794 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of the nasopharyngeal washings from Case 11. Feb. 20. No change. Feb. 21. Conjunctivitis. Temperature 40.5°C. Leucocytes decreased to 6,100; mononuclears to 2,989. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—No growth.

*Second Passage. Rabbit B.*—Feb. 21, 1919. Leucocytes 12,650, of which 6,451 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit A. On the next 2 days there were fever (41.3° and 40.6°C.), leucopenia (leucocytes 5,760 and 5,775), and mononuclear depression to 2,592 and 1,098 cells. Feb. 23. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—No growth.

*Third to Eighth Passages. Rabbits C, D, E, F, G, and H.*—These rabbits developed, 24 hours after the intratracheal injection of the suspensions of lung tissue from the immediately preceding rabbit in the series, a pronounced leucopenia (in one case a decrease in leucocytes from 15,900 to 2,350), mononuclear depression (in Rabbit D, for example, from 7,632 to 940 cells), conjunctivitis, and fever (usually above 40.2°C.). The rabbits of the third, fourth, and fifth passages died 48 hours after injection; those of the sixth, seventh (Text-fig. 1, c), and eighth were killed in the usual manner.

*Autopsy.*—In all instances definite areas of consolidation were found in one or more lobes, while elsewhere the lung contained the typical areas of hemorrhage and edema accompanied with emphysema.

*Aerobic Cultures.*—The consolidated foci of the third, fourth, and fifth passages yielded *Pneumococcus* Type IV, and of the sixth, seventh, and eighth an atypical Type II pneumococcus.

*Ninth Passage. Rabbit I.*—Mar. 5, 1919. Leucocytes 14,225, of which 7,397 were mononuclears. Temperature 39.5°C. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit H. After 48 hours the temperature rose to 40°C., the leucocytes diminished to 10,225, of which 6,237 were mononuclears. This persisted for 2 days. Mar. 9. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—No growth.

*Tenth Passage. Rabbit J.*—Average leucocyte count prior to inoculation was 15,353, of which 7,587 were mononuclears. Temperature 39.6°C. Mar. 9, 1919. Injected intratracheally with 3 cc. of the suspension of lung tissue from Rabbit I. After 24 hours, and for the next 3 days, the temperature rose to 40°C., the leucocytes diminished to 11,400, reaching 8,800 on the 3rd day, and the mononuclears decreased to an average of 4,886 cells. Mar. 13. Killed.

*Autopsy.*—Lungs showed the typical lesions.

*Aerobic Cultures.*—No growth.

With this, the tenth rabbit passage, the series was discontinued.

This series closely reproduces the previous one. The transmission experiments were discontinued with the tenth passage and at a time when the typical effects were still being produced regularly. Several instances of fatal intercurrent infection were encountered, the pneumonia present being associated with *Pneumococcus* Type IV or atypical Type II. But other portions of the lungs, which were free of these and other ordinary bacteria, were suitable for the transmission experiments.

#### PROTOCOL 5.

*Patient 17.*—S. C., adult male. Apr. 10, 1919. Sudden onset at night with symptoms of languor, chills, headache, and dull pain in the back. Apr. 11. General malaise; prostration; fever; headache; no cough or rhinitis. Physical examination showed conjunctivæ injected, pharynx congested, and lungs clear (also by radiographic examination). Other organs unaffected. Leucocytes 5,875, of which 2,059 were mononuclears. Blood culture sterile. Sputum not obtainable. A throat culture yielded no Pfeiffer bacilli on oleate agar (Avery medium<sup>12</sup>), but a Gram-negative coccus on blood agar plates. Nasopharyngeal washings yielded this coccus and *Streptococcus viridans*. On the 3rd day of illness the temperature was normal, and an uneventful recovery followed.

*Animal Inoculation.*—The unfiltered nasopharyngeal washings obtained 12 hours after the onset of symptoms were inoculated intratracheally into Rabbit A.

*First to Third Passages.* *Rabbits A, B, and C.*—These animals were employed for the first, second, and third passages respectively, the same methods being used as those described in the previous transmission experiments. Rabbit A died over night, Rabbit C after 2 days, and Rabbit B was killed. In all instances fever (above 40°C.) developed, with conjunctivitis and a prompt and marked leucopenia and mononuclear depression similar to those already given.

*Autopsy.*—The lungs revealed typical hemorrhagic edema, the hemorrhages varying from diffusely scattered small foci to large areas involving almost an entire lobe, sometimes of infarct shape. There was no definite consolidation.

*Aerobic Cultures.*—The lungs of the three animals yielded Type IV pneumococci.

*Fourth to Fifteenth Passages.*<sup>13</sup>—These were initiated with a filtrate, free from aerobic bacteria, of the suspension of lung tissue from Rabbit C.

In this experiment the pneumococcus present in the nasopharyngeal secretions was not suppressed during the first to third rabbit passages.

<sup>12</sup> Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

<sup>13</sup> These experiments will be described in detail in a later communication.

But the effects in the animal, even though severe and fatal, bore so close a resemblance to those arising in the four previous series of rabbits that it was deemed advisable to free the material from the pneumococcus by filtration. The filtrate thus secured, free from aerobic organisms, was passed through a series of twelve animals in which the typical clinical and pathological effects were obtained.

Thus far we have dealt with cases of influenza which occurred during the epidemic of 1918-19. The next series of experiments relates to cases arising in the winter of 1920 of which three uncomplicated cases in the early stage of the affection were available for study.

#### PROTOCOL 6.

*Patient 24.*—H. J., adult female. Jan. 20, 1920. Onset sudden with chills and fever. Jan. 21. Prostration; photophobia; muscular pains; unproductive cough. Conjunctivitis and injected pharynx; lungs negative. Leucocytes 2,800, of which 336 were mononuclear. Cultures from the nasopharyngeal washings yielded numerous colonies of *Staphylococcus albus* and *Micrococcus flavus*, and a moderate number of Pfeiffer bacilli.

*Animal Inoculation.*—The filtered and unfiltered washings obtained 20 hours after the onset were inoculated into the lungs of three rabbits which, within 24 hours, developed conjunctivitis, fever, leucopenia, and mononuclear depression similar to those in the other instances cited. The animals were killed 48 hours after inoculation.

*Autopsy.*—The lungs showed the typical hemorrhagic, emphysematous, and edematous condition.

With the lungs of these rabbits another series of transmissions, consisting of three rabbits, two for the filtered material, to be described in another communication, and one for the unfiltered, was carried out. The results in each case were typical.

#### PROTOCOL 7.

*Patient 26.*—M. O., adult female. Feb. 4, 1920. Onset sudden with chills and fever. Temperature 40.1°C. Feb. 5. Fever; muscular pains; unproductive cough. Conjunctivitis; lungs clear. Leucocytes 6,000, of which 1,380 were mononuclears. Cultures from the nasopharyngeal washings yielded mainly *Staphylococcus albus* and also pneumococci, hemolytic streptococci, and occasional Pfeiffer bacilli.

*Animal Inoculation.*—The filtered and unfiltered nasopharyngeal washings obtained 30 hours after the onset were injected intratracheally into Rabbits A, B, and C, the last two receiving the filtrates. These animals showed the typical clinical and pathological effects, similar to those of the rabbits described in Protocols 1 and 2. No ordinary bacteria were detected in cultures or films of the lungs. The rabbits were killed 48 hours after the inoculation.

With the lungs of these animals another series of transmissions was carried out, with results which were regarded as typical. The gross appearance of the lungs of one rabbit is shown in Fig. 2. The lungs of one rabbit of the first passage of the filtered material (Rabbit C) were preserved in sterile 50 per cent glycerol for 4 months, and the lungs of Rabbit D, the second passage of the unfiltered washings, for 10 days. With these glycerolated lungs further transmissions were carried out to be described in another communication, until the experiment was voluntarily discontinued with the sixth rabbit passage. All showed the typical clinical and pathological effects, and in no instance were ordinary bacteria detected in cultures or in stained films from the lungs.

#### PROTOCOL 8.

*Patient 27.*—P. S., adult male. Feb. 9, 1920. Onset sudden with chills and fever. Feb. 10. Fever; muscular pains; sore throat; tracheal cough; headache; prostration. Lungs clear. Leucocytes 13,000, of which 10,140 were polymorphonuclears. Cultivation of nasopharyngeal washings yielded an almost pure culture of hemolytic streptococci and a few colonies of Pfeiffer bacilli. The question was raised of a complicating streptococcus sore throat in this patient.

*Animal Inoculation.*—The filtered and unfiltered nasopharyngeal washings obtained 20 hours after onset were injected into the lungs of five rabbits. The filtrates produced no effect; the unfiltered material caused abscess of the lung, from which hemolytic streptococci were isolated. The material was not inoculated further.

It is seen that the nasopharyngeal secretions of two of three cases of influenza during the second or 1920 epidemic gave rise in rabbits to clinical and pathological effects, independently of the presence of ordinary bacteria, similar to those obtained during the first or 1918 epidemic.

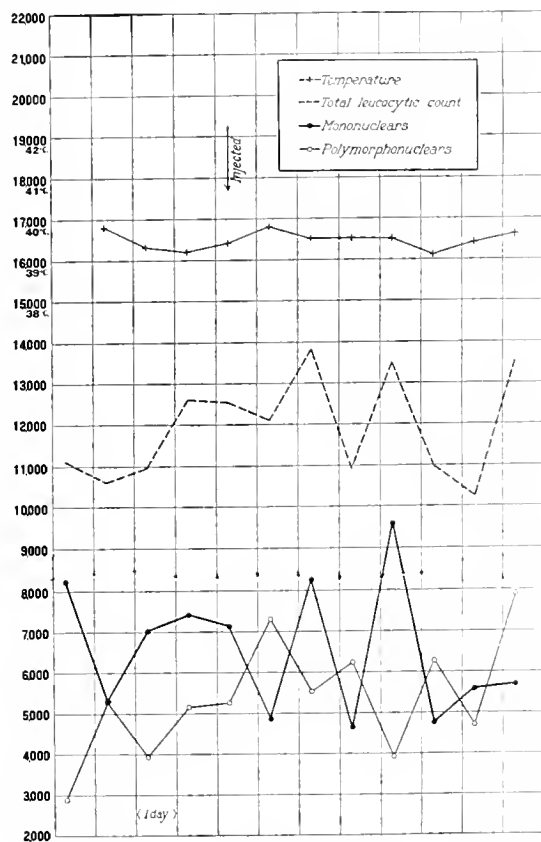
#### *Negative Transmission Experiments.*

In addition to the positive transmission experiments presented under Protocols 1 to 8, several cases of influenza, at somewhat later stages in the evolution of the disease, were studied in the same manner. The cases from the 1918 epidemic included five which were in the 3rd day of the disease when the washings were secured, four in the afebrile and convalescent stage, and two in course of a secondary pneumonia. From the 1920 epidemic one case only, on the 1st afebrile day, was studied.

The washings from the nasopharynx of these individuals were collected and injected into rabbits in the manner already described, and



in none of them was the characteristic effect on the blood count observed (Text-fig. 2). When a blood change did occur it was of the nature of a polymorphonuclear leucocytosis. The lung lesions, when any were present, consisted of lobar consolidation in which pneumococci, as a rule, were demonstrated.

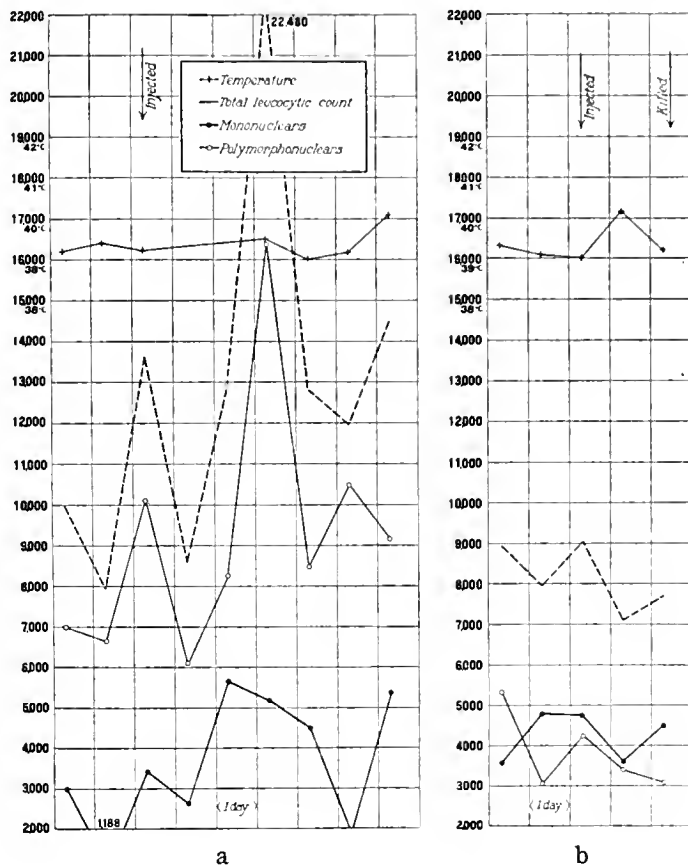


TEXT-FIG. 2. First rabbit passage of the nasopharyngeal washings from Patient 5. The washings were obtained at the beginning of the 3rd day of uncomplicated influenza. No effect on blood count and temperature.

#### *Control Experiments (Text-Fig. 3, a and b).*

The control tests consisted of the injection into the lungs of rabbits of saline solution, suspensions of normal rabbit lungs, normal rabbit serum, foreign protein, such as human ascitic fluid, bacteria of the

ordinary species,<sup>14</sup> including Pfeiffer's bacillus and its poison as prepared by Parker's method,<sup>15</sup> and finally, the nasopharyngeal secretions from fourteen persons free from influenza and tested in the epidemic



TEXT-FIG. 3, *a* and *b*. Effect on the blood count and temperature of intratracheal inoculations of control materials. (*a*) Inoculation of nasopharyngeal secretions from a normal individual free from an influenzal attack. A transient polymorphonucleosis on the 3rd day after inoculation is shown. (*b*) Inoculation of a suspension of normal rabbit lung. No effect on blood count and temperature.

<sup>14</sup> Control experiments with ordinary bacteria injected intratracheally will be described in another communication.

<sup>15</sup> Parker, J. T., *J. Immunol.*, 1919, iv, 331.

and interepidemic periods. Of the latter, seven suffered from early or later stages of coryza. The lung tissue of the inoculated rabbits was in turn reinoculated into two successive series of rabbits. None of the 55 animals inoculated with the control materials or these secretions showed the familiar clinical and pathological action; a few gave a polymorphonucleosis with frank lobar pneumonia, others a mononucleosis without lung involvement, and still others inconstant effects.

#### DISCUSSION.

The object of the present investigation was to determine, if possible, whether the secretions of the nasopharynx of individuals suffering from epidemic influenza exhibited on inoculation into animals any peculiarities of action or properties which would serve to distinguish them from the secretions of individuals not so affected. Obviously, the first requisite was a standard by means of which this action, or effect, could be detected. We sought one which was subject to measurement with at least a fair degree of accuracy and did not necessitate killing the inoculated animal. This criterion was found in the blood, associated with changes in the absolute and differential white blood count and correlated with the leucocytic curve in uncomplicated cases of epidemic influenza in man. A second criterion, observable after the animal had been killed, was discovered in certain hemorrhagic, edematous, and emphysematous changes in the lungs.

The next point was the determination of the relation of the changes noted in the leucocyte count and the lung structures to the ordinary bacterial flora of the nasopharynx. The fact that the changes were regularly observable, under favorable conditions, in the complete absence of ordinary bacteria in culture and in film, was regarded as particularly significant.

The deduction arrived at, therefore, as a result of the experiments carried out in rabbits was to the effect that patients with epidemic influenza, within at least the early stage of the obvious infection, carry in their nasopharyngeal secretions a substance which is not ordinary bacteria or their metabolic products. This substance when inoculated intratracheally into rabbits readily causes fever, leucocytic and particularly mononuclear cell depression, lung hemorrhage, edema, and em-

physema. Whatever this active substance is, it seems to disappear from or to diminish in the nasopharyngeal secretions of cases of epidemic influenza so as to be no longer discoverable by inoculation tests about 36 hours after the obvious symptoms of the disease have appeared, and to be absent from healthy persons and in other pathological conditions.

When implanted in the lungs of the rabbit this substance appears to increase, since it remains active through a long series of inoculations of the lung tissue in successive passages through rabbits.

The substance is readily filterable through Berkefeld filters. It is capable of surviving and apparently of multiplying in association with ordinary bacteria not only in the nasopharyngeal secretions in man but also in the lungs of rabbits, in the latter at least for a time.

When the unfiltered washings containing nasopharyngeal secretions of patients in early stages of epidemic influenza are injected intratracheally into rabbits, this substance when present exerts its peculiar action while some multiplication of ordinary bacteria (usually *Pneumococcus* Type IV) is going on in the lung. The successive passage of unfiltered emulsions of selected parts of the lungs, away from obviously infected and consolidated areas, leads often to rapid and complete disappearance of the ordinary bacteria and survival and possibly increase of this active substance.

No attempt will be made in this paper to define further the nature of the active substance or to relate it more accurately and specifically with the etiology of epidemic influenza.

#### SUMMARY.

An active substance has been detected, by the methods described, in five patients in early stages of epidemic influenza during 1918-19 and two patients in early stages of epidemic influenza during 1920. It was not detected in twelve cases of the same disease in which the onset of obvious symptoms occurred more than 36 hours before washing of the nasopharynx was carried out, nor was it found in the secretions of fourteen individuals free from the syndrome of influenza either during the epidemics or the interval between them.

With this substance a clinical and pathological condition has been induced in rabbits, affecting the blood and pulmonary structures

mainly, which could be maintained and carried through at least fifteen successive animals. For this reason, and also because of the dilution between passages, we are led to believe that we were dealing with the actual transmission of a multiplying agent rather than with a passive transference of an original active substance.

In some of the experiments secondary infections by ordinary bacteria were encountered. The relation of these microorganisms to this active substance will be dealt with fully in another communication. However, the essential effects were produced by a substance wholly unrelated to these bacteria.

The similarity that exists between the effects produced in rabbits on the blood and the lungs and those occurring in man in epidemic influenza provides a basis for further investigation on the inciting agent of epidemic influenza.

#### EXPLANATION OF PLATES.

##### PLATE 5.

FIG. 1. Gross lesions of a lung from Rabbit F, representing the sixth rabbit passage of the nasopharyngeal secretions from Patient 16. The hemorrhages, edema, and emphysema of the lung, more marked in the upper lobe, and absence of pneumonic consolidation are noteworthy. Natural size.

FIG. 2. Gross lesions of the lungs from Rabbit D, representing the second rabbit passage of the nasopharyngeal secretions from Patient 26. This case occurred in the second epidemic of 1920 and is to be compared with Fig. 1 derived from the first epidemic of 1918-19. Hemorrhages, edema, and emphysema are shown. A small area of atelectasis is seen at the inner margin of the lower right lobe. Natural size.

##### PLATE 6.

FIG. 3. Microscopic appearance of a section of the lung shown in Fig. 1. Edema and emphysema are present. A vessel is shown distended with blood, and mononuclears may be seen in the intervalveolar tissues.  $\times$  about 190.

##### PLATE 7.

FIG. 4. A different section of the same lung. The small discrete hemorrhages, the edema, and the cellular exudate are shown.  $\times$  about 190.



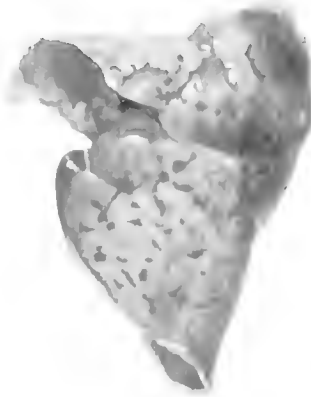


FIG. 1.

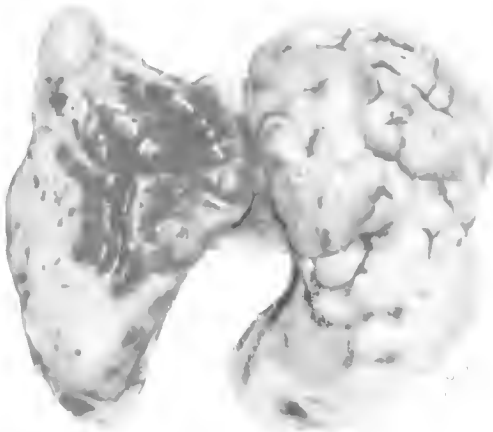


FIG. 2.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. 1.)





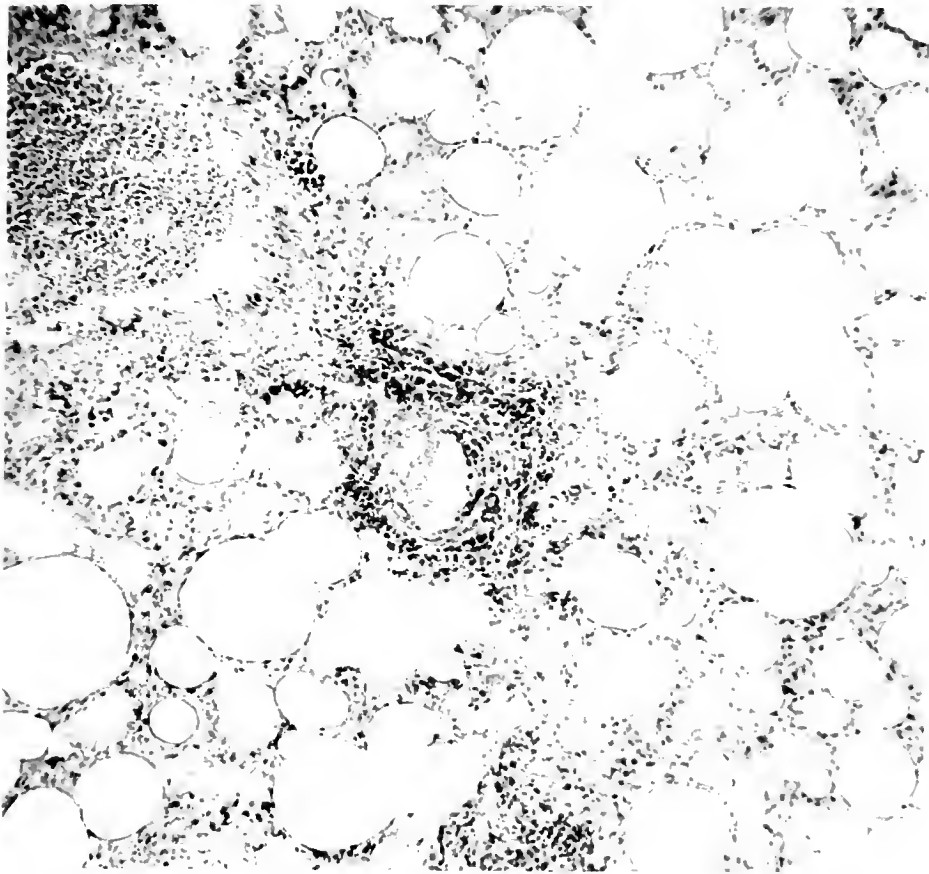


FIG. 3.



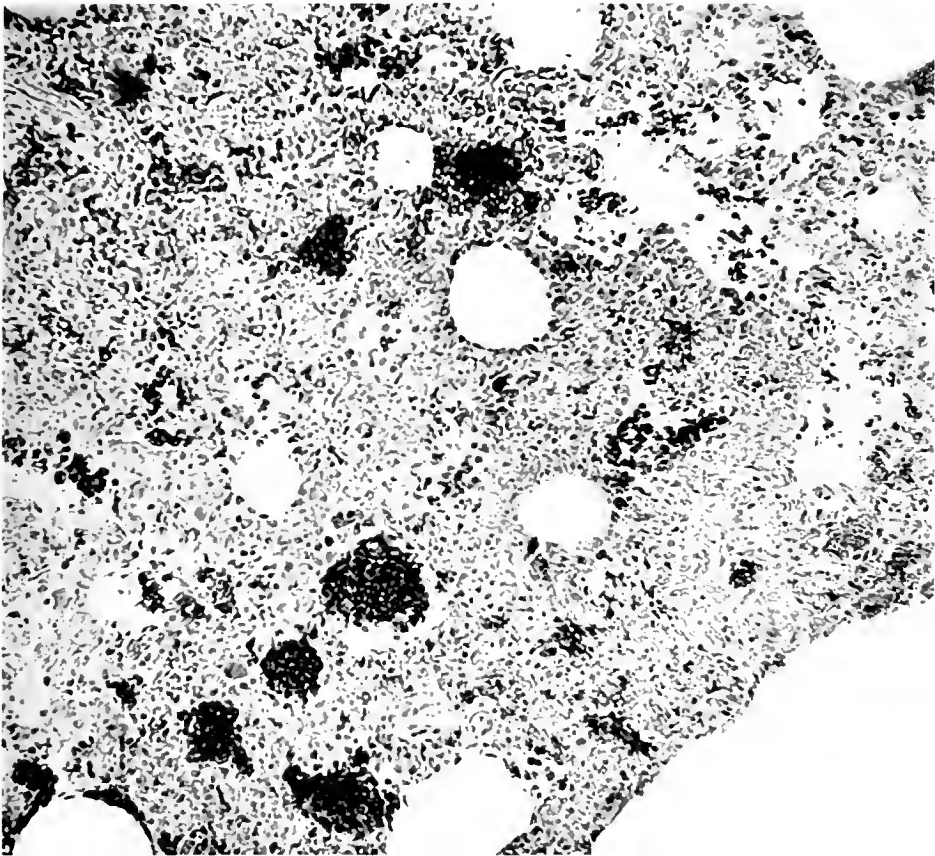


FIG. 4.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. 1.)



## CHILOMASTIX MESNILI AND A METHOD FOR ITS CULTURE.

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(Received for publication, July 26, 1920.)

In January, 1920, a chronic case of diarrhea due to a flagellate infection was brought to the notice of the author and upon examination the flagellate was identified as *Chilomastix mesnili*. Attempts to culture this parasitic flagellate proved successful. Observations made from time to time upon the flagellates in both the stools of the patient and the cultures, as well as data derived from a study of permanent preparations, have revealed further information regarding the biology of this organism. It is the purpose of this paper to describe this parasite and its activities and to give a method of culture for the growth of this organism in artificial medium.

Regarding its phylogeny, *Chilomastix mesnili* belongs to the family Tetramitidae, the order Polymastigina, and the class Mastigophora.

The habitat of the parasite is the small intestine of man. It is often the cause of chronic or intermittent diarrhea. Cases of infection by *Chilomastix* in man have been reported from nearly every locality in the world. Chalmers and Pekkola<sup>1</sup> state that they have always found *Chilomastix* associated with other protozoa and not entirely by itself. But in the case of infection referred to above *Chilomastix* was the only protozoan parasite found. In all probability it is the cause of the patient's diarrhea. No formed stools have been passed since the discovery of the infection 2 years ago which was then thought to be caused by *Trichomonas intestinalis*. In the United States, cases of infection by *Chilomastix mesnili* are

<sup>1</sup> Chalmers, A. J., and Pekkola, W., *Chilomastix mesnili* (Wenyon 1910), *Ann. Trop. Med. and Parasit.*, 1918, xi, 213.

by no means rare, for Kofoid, Kornhauser, and Plate<sup>2</sup> found it present in 5.8 per cent of the 1,200 overseas troops which were examined at New York. Of 534 persons examined at Berkeley, California, by Kofoid and Swezy,<sup>3</sup> 28, or 5.3 per cent, were found infected. Many clinicians no doubt have confused *Chilomastix mesnili* with *Trichomonas intestinalis*. This mistake is due to the similarity in movement of the cytostomal flagellum of *Chilomastix mesnili* to the undulating membrane of *Trichomonas intestinalis*.

### Morphology.

The cultivation of *Chilomastix mesnili* in artificial medium has made observations possible from day to day. Under these cultural conditions the flagellates grew and multiplied. Apparently the environment satisfied most of the biological requirements of the parasites. The data given here regarding the morphology and activity of these organisms are based upon direct and continued observations made upon the flagellates under cultural conditions, and also upon a study of permanent preparations made from the cultures and from the feces of the patient harboring the flagellates.

*Motile Flagellate.*—The location of the various organs in *Chilomastix* will be given as they are seen in a dorsal view, for it is from the dorsal view that convention or custom has always designated the right and left sides of any object.

The best descriptions of the motile or active form of *Chilomastix mesnili* have been supplied by Chalmers and Pekkola<sup>1</sup> and more recently by Kofoid and Swezy.<sup>3</sup> The descriptions given by these two groups of workers differ in some respects; that of Kofoid and Swezy,<sup>3</sup> however, is the more valuable because details of morphology are given which were previously unknown. The description given here corroborates that of Kofoid and Swezy<sup>3</sup> in nearly all details. The differences of interpretation and interpolation of new data regarding the morphology and the activities of the flagellates have come as a result of continued study of the organisms in cultures over a period

<sup>2</sup> Kofoid, C. A., Kornhauser, S. I., and Plate, J. T., Intestinal parasites in overseas and home service troops of the U. S. Army, *J. Am. Med. Assn.*, 1919, lxxii, 1721.

<sup>3</sup> Kofoid, C. A., and Swezy, O., On the morphology and mitosis of *Chilomastix mesnili* (Wenyon), *Univ. California Pub., Zool.*, 1920, xx, 117.

of 4 months. The cultures were inoculated on January 27, 1920, and were kept alive by subculturing until June 15, 1920, when a mishap to the heating apparatus of the incubator caused it to reach 80°C., which resulted in the death of the organisms.

The usual shape of the body of *Chilomastix mesnili* is pyriform with a rounded anterior end and a pointed posterior end terminating in a spine-like process. The latter structure is composed almost entirely of hyaline periplast. The sides begin to taper posteriorly at the middle of the body. This tapering is not uniform for both sides but varied, depending upon the particular state of locomotion the flagellate may be in at any given moment. It is not uncommon to find forms which have the ventrum flattened, and the dorsum arched, so that in profile they resemble a side view of *Giardia intestinalis*. When these particular flattened forms were encountered, the posterior spine appeared as a posterior terminal extension of the ventrum, while in other forms the spine is terminal but more axially situated.

In the stools, the active flagellates have been seen to assume the shape of a sphere, and the spine-like protuberance has been withdrawn by contraction. On two occasions twisted forms have been seen similar to those described by Wenyon and O'Connor,<sup>4</sup> Chalmers and Pekkola,<sup>1</sup> and Kofoid and Swezy.<sup>3</sup> The twisted forms have not been encountered in the cultures. Fortunately, it was possible to observe these atypical flagellates for a considerable length of time and evidence was obtained which may explain their twisted condition.

The twisted state of the flagellate appears to be a temporary condition, at least as far as the flagellates in this particular case are concerned.

Wenyon<sup>5</sup> did not note this twisted condition in his original description of *Chilomastix mesnili* from man, and it was not until later that he and O'Connor<sup>4</sup> described it. Chalmers and Pekkola<sup>1</sup> spoke of this twisted condition as due to "a dorsal twisting of the antero-ventral part, while the posterior part of the body is not affected. This twist naturally gives rise to the appearance of lines and

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<sup>4</sup> Wenyon, C. M., and O'Connor, F. W., Human intestinal protozoa in the Near East, London, 1917.

<sup>5</sup> Wenyon, C. M., A new flagellate (*Macrostoma mesnili* n. sp.) from the human intestine with some remarks on the supposed cysts of *Trichomonas*, *Parasit.*, 1910, iii, 210.

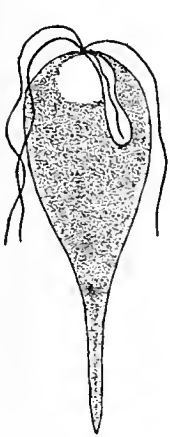
grooves running more or less diagonally backwards across the body." Kofoid and Swezy<sup>3</sup> found that this twisted condition, resulting in a diagonal groove, characterized most of the active forms of *Chilomastix mesnili* and therefore concluded that the groove was a permanent structure. They also believed that in the forms where the groove was not in evidence, it had temporarily disappeared due to the contraction of the body.

Since January, 1920, the author has received fecal specimens once or twice each week from the patient infected with *Chilomastix mesnili*. As has been said before, on only two occasions were the twisted forms encountered. On these occasions it was also found that the flagellates were definitely changed in shape. The anterior end was rounded as usual but the posterior half of the body was broadly flattened from side to side. A dorsal view showed the body almost truly pyriform with uniformly tapering sides (Text-fig. 1). A lateral view resembled the side view of a tadpole, for the posterior end possessed a broad flattened tail instead of a pointed spine (Text-fig. 2, *a*). On closer observation it was seen that the tail contained two rib-like extensions of the granular endoplasm running out into the hyaline periplast of the ectoplasm. These two ribs of endoplasm were united anteriorly where they joined the mass of endoplasm of the anterior portion of the body, but they began to diverge as they proceeded posteriorly and became separated by a thin membrane-like structure made only of the periplast. The area of the tail, constituted only of the membrane of periplast between the two ribs of endoplasm, was thinner than the adjacent areas occupied by the endoplasmic ribs, which were also surrounded by periplast. This condition resulted in the formation of a straight groove on each side of the body between these extensions of the endoplasm (Text-fig. 2, *b*).

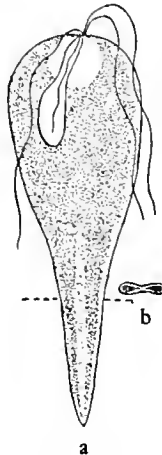
These forms, in addition to being laterally flattened, assumed the twisted condition both when at rest and during locomotion. They possessed definite grooves running diagonally and posteriorly from the left to the right side (Text-fig. 3). To all appearances these flagellates were similar to the twisted forms described by Wenyon and O'Connor,<sup>4</sup> Chalmers and Pekkola,<sup>1</sup> and somewhat like those described by Kofoid and Swezy.<sup>3</sup> In view of the presence of the laterally flattened forms which showed grooves in the posterior region, but were not twisted, it seemed plausible that the grooves of the



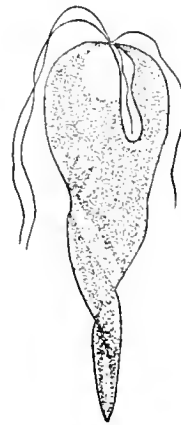
twisted forms were identical with those of the flagellates which were not twisted, and that this state was a temporary condition brought about by some factor which accounted for the change in form of the flagellates. The flagellates which were not twisted but were flattened may either have recovered from the twisted condition or not as yet become so transformed. The temporary nature of the twisted condition of the flagellates of this particular case of infection seems all the more substantiated since it was never observed in the flagellates which have been growing and reproducing in cultures for over 4 months.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 1. *Chilomastix mesnili*, dorsal view, unstained. Posterior region compressed.  $\times 3,300$ .

TEXT-FIG. 2. *Chilomastix mesnili*, unstained. (a) Lateral view showing compressed tail; (b) cross-section of tail to show grooves.  $\times 3,300$ .

TEXT-FIG. 3. *Chilomastix mesnili*, dorsal view, unstained. Twisted form showing diagonal groove.  $\times 3,300$ .

The motile organisms observed in this case measured 3 to 19 microns in length and 2 to 9 microns in width. Most forms in the stool were 8 to 14 microns in length. The flagellates possessed three anterior flagella and a cytostomal flagellum situated in the oral cavity, or cytostome. In a dorsal view (Text-fig. 4) the cytostome is located on the ventrum and the right side, extending backward a distance of one-third to one-half the length of the body. The nucleus

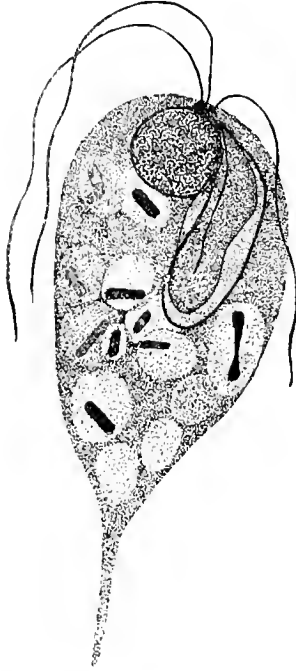
is round, and located near the anterior border of the body, to the left of the cytostome and dorsal to it. In unstained specimens it appears somewhat refractile. The cytoplasm is granular and generally contains large vacuoles, in many of which was a bacterial cell which had been previously ingested as food. At the times when binary fission is in process a tendency probably exists on the part of the flagellate to desist from eating, for in many of the forms undergoing division no vacuoles containing bacteria were seen.

In locomotion, *Chilomastix mesnili* is propelled forward and spirally in a jerky manner by the action of its flagella. The two left anterior flagella, as Kofoid and Swezy<sup>3</sup> have pointed out, beat backward and against the left side of the body, and the other anterior flagellum beats backward against the right side of the body. The motion of these flagella is undulatory, the ripples starting at the point of origin of the anterior flagella and proceeding posteriorly. These waves of contraction diminish as they progress distally along each flagellum.

The cytostomal flagellum lies within the cytostome and forms an undulating membrane by adhering to the inner portion of the cytoplasmic lip or membrane which is also within the cytostome (Text-fig. 4). This flagellum is probably not involved in any purpose of locomotion but functions chiefly as a means of obtaining food. Gäbel<sup>6</sup> and Chalmers and Pekkola<sup>1</sup> noted that the flagellum may not always form an undulating membrane but may act as a free flagellum. In the writer's observations upon living forms thus far the flagellum has not been seen as a free whip but always combined with the thin cytoplasmic lip to form an undulating membrane. In the flagellates more or less quiescent, the action of this cytostomal undulating membrane was easily observed. The undulating waves of contraction began at the anterior end of the cytostome at the place of origin of the cytostomal flagellum and proceeded posteriorly to the end of the cytostome. The initial wave of contraction of the undulating membrane was very large, for at times the membrane was pushed outward from the cytostome 3 to 4 microns. The amplitude of this first wave of contraction diminished as the wave progressed posteriorly. The action of the undulating membrane had the effect of

<sup>6</sup> Gäbel, M., Zur Pathogenität der Flagellaten, *Arch. Protistenk.*, 1914, xxxiv, 1.

reaching out into the surrounding medium and literally scooping the bacteria toward the cytostome. In this way food was brought in reach of the cytostome. The anterior flagellum which beat backward against the right side was very close to the cytostome and aided materially in setting up a current in the medium which helped to bring the food in proximity to the cytostome



TEXT-FIG. 4. *Chilomastix mesnili*, dorsal view, stained. Taken from culture.  $\times 4,400$ .

The cytostome has had holdfast properties attributed to it by Kofoid and Swezy.<sup>3</sup> No doubt because of its size and morphology it may serve such a purpose, but in the study of the flagellates in the cultures, as well as in fecal specimens, the posterior spine or protuberance was used for this purpose almost entirely.

When the flagellate was feeding, it inserted this posterior spine into a mass of bacteria, while its body remained free from any encumbrance. In this position the flagellate anchored itself and proceeded

to rotate, using the posterior spine as the holdfast organ. On such occasions one could watch the undulating membrane as it wafted bacteria into the cytostome. Not all the bacteria which were taken into the cytostome were ingested, for many of them could be seen escaping in a current in the medium directed away from the cytostome. It is very likely that the flagellate exercised a selective sense in the choice of its food.

The finer details of morphology have been described by Kofoid and Swezy.<sup>3</sup> They have shown *Chilomastix mesnili* to possess an elaborate neuromotor apparatus. The author has been able to verify the finding of the structures described by these workers, but because the finer morphological structures which only become apparent in properly prepared, stained specimens have received a full description by the above investigators, brief reference is made to them in this paper.

The nucleus is round and possesses a distinct membrane (Text-fig. 4). It is located near the anterior border of the body to the left of, and dorsal to the cytostome. The chromatin may be distributed in a variety of ways, but it is often in the form of two terminal masses lodged against the nuclear membrane with a small eccentrically placed karyosome, or broken up into chromidia distributed diffusely throughout the nucleus. The chromatin was also resolved into a definite spireme strand, which in some forms had later segmented to form chromosomes.

A very small centrosome is located at the anterior pole of the nucleus (Text-fig. 4). It is often concealed by a larger chromatin-staining mass, the blepharoplast complex. As Chalmers and Pekola,<sup>1</sup> and Kofoid and Swezy<sup>3</sup> have shown, there are three blepharoplasts which are only seen as separate granules in well decolorized preparations. The primary blepharoplast lies closest and anterior to the nucleus. It gives origin to the pair of anterior flagella which beat backward against the left side of the body. The secondary blepharoplast lies farther to the right side and gives rise to the right anterior flagellum. Kofoid and Swezy<sup>3</sup> found that this secondary blepharoplast also gives rise to the parastyle, a slender, slightly curved rod, which extends posteriorly in the left wall of the cytostome. The tertiary blepharoplast lies at the anterior end of the

cytostome and gives rise to the cytostomal flagellum, the peristomal fiber, and the parabasal. All the blepharoplasts are joined together in series with the centrosome and often with the anterior terminal mass of chromatin of the nucleus by fine rhizoplasts. In many forms the primary and secondary blepharoplasts are fused into a single large granule located against the nuclear membrane at the anterior pole of the nucleus (Text-fig. 4). This double blepharoplast by virtue of its position often obscures the centrosome.

The peristomal fiber borders the inner free edge of the membranous lip of the cytostome. It is a fine fibril originating from the tertiary blepharoplast. The cytostomal flagellum when in action appears to adhere to the inner portion of this loose membranous lip to form an undulating membrane within the cytostome, or oral pouch. That this is the case is evidenced by the location of the cytostomal flagellum lying along the side of, and adhering to the inner portion of this lip in many stained specimens. Furthermore, in moribund flagellates it is the inner portion of the lip which is seen to push out for a great distance to begin a wave of contraction which lessens and dies out as the contraction moves posteriorly. In other cases the cytostomal flagellum may be seen to lie on the floor of the cytostome (Text-fig. 4) or at times just under the inner portion of the peristomal fiber.

The parabasal is a large chromatin-staining rod extending from the tertiary blepharoplast along the right side and around the posterior end of the cytostome. In some forms it appears to surround completely the cytostome. However, Kofoed and Swezy<sup>3</sup> found that in such cases, if the preparations have been well destained, this rod is not continuous but is composed of two elements, the parabasal, extending from the tertiary blepharoplast along the right side and around the posterior end of the cytostome, and another rod, the parastyle, arising from the secondary blepharoplast and extending along the left side of the cytostome to within a very short distance of the posterior end of the parabasal. Chalmers and Pekkola<sup>1</sup> believed the parabasal was a continuous rod arising from the secondary blepharoplast and returning to it after it had run a circuitous course around the cytostome.

Kofoed and Swezy<sup>3</sup> have shown the homology which exists between the peristomal fiber and parastyle of *Chilomastix mesnili* with the

peristomal fiber and the right axostyle of the right half of *Giardia*. There is good morphological evidence to draw a homology between the peristomal fibers of *Chilomastix* and *Giardia*, and likewise between the parastyle of the former with the right axostyle of the latter organism, but there remains the problem of finding a homologue in *Giardia* for the parabasal of *Chilomastix*. It is more than likely that the parabasals of *Giardia* differ so much in structure and chemical constitution that they cannot be considered homologues of the parabasal of *Chilomastix*. On the other hand, Chalmers and Pekkola<sup>1</sup> showed the parabasal of *Chilomastix* to be homologous to the parabasal of *Trichomonas*, since in both these organisms this rod has the same morphological relation to the undulating membrane. In view of these relations existing between the structures of *Chilomastix*, *Trichomonas*, and *Giardia*, *Chilomastix* may be said to occupy a position midway between the trichomonad flagellates and members of the genus *Giardia*. *Chilomastix* may have resulted as an offshoot from the trichomonad flagellates, and, as Kofoid and Swezy<sup>3</sup> have pointed out, it may be the form from which *Giardia* and other Hexamitidæ have originated.

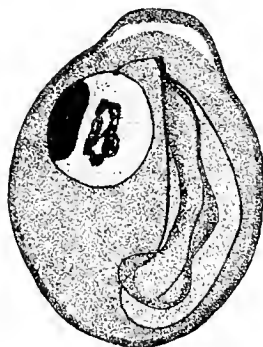
*Cysts*.—The cysts of *Chilomastix mesnili* are usually found in varying numbers along with the active flagellates. However, they may be entirely absent from a fecal specimen containing an abundance of motile organisms. This fact is evidence of encystment being an irregular if not a cyclic process dependent perhaps on many causes. Wenyon and O'Connor,<sup>4</sup> in the case of a patient undergoing treatment for *Entamoeba histolytica*, record the absence of the cysts of *Chilomastix* for 20 days; the flagellates, however, were present on 13 days of this period. In the fecal samples received for examination once or twice each week since January, 1920, the cysts have been absent on only a few occasions.

The cysts are usually somewhat lemon-shaped, with one end rounded and the other end terminating in a slight protuberance (Text-fig. 5). Occasionally they are ovoid and lack the terminal process. In size, the cysts measure 4.5 to 6 microns in width and 7.6 to 9 microns in length. Wenyon and O'Connor<sup>4</sup> record cysts with a length of 10 microns.

The wall of the cyst is transparent and of uniform thickness except in the vicinity of the terminal elevation where it is thicker than at other places.

The cytoplasm within the wall of unstained cysts is refractile and structureless except for the presence of a few bright spots. With favorable lighting facilities for the microscope, a dull brownish area can be seen which is the nucleus. There is a space between the cytoplasm and the cyst wall immediately back of the terminal protuberance.

In stained specimens, the cytoplasm appears to be very finely granulated. At one side toward the anterior end is a large round or ovoid nucleus. The chromatin is often distributed in the form of one large karyosome with accessory chromatin matter, or as a central



TEXT-FIG. 5. *Chilomastix mesnili* cyst, stained.  $\times 4,400$ .

karyosome with one or two terminal masses, or broken up into chromosomes. Kofoed and Swezy<sup>3</sup> have described in detail the mitosis which takes place within the cyst. These investigators were the first to find cysts containing two nuclei and two sets of neuromotor apparatus. The karyosome of the nucleus is often connected by a rhizoplast with the centrosome on the nuclear membrane at the anterior pole of the nucleus. The centrosome is in turn connected with the blepharoplasts by fine rhizoplasts (Text-fig. 5). In many cysts, as in the case of the motile flagellates, the distinct entity of all three blepharoplasts cannot be made out, only two masses being apparent. The primary and secondary blepharoplasts have become united into a single large granule. The tertiary blepharoplast remains separate at the anterior end of the cytostome.

The entire structure of the neuromotor apparatus of the motile organism is preserved and carried over into the cyst. It is possible in properly stained specimens to locate the peristomal fiber, the parabasal, the parastyle, and the cytostomal flagellum. In many cysts, however, some members of this fibrillar apparatus are not discernible. The failure to see these structures may be due to imperfect fixation and decolorization after staining or the structures may have actually disintegrated.

### *Method of Culture.*

*Medium.*—Less difficulty was experienced in culturing *Chilomastix mesnili* in artificial medium than was anticipated. There were only two fundamental substances used in the preparation of the culture medium; namely, blood serum and a physiological solution. Three kinds of blood serum, horse, sheep, and human, were combined with varying proportions of either Ringer's or Locke's solution. In the attempt to find a suitable medium, the proportions of blood serum and the physiological solutions were varied from equal parts of both to one part of blood serum and two to seven parts of the Ringer's or Locke's solution. But after trying out these various combinations, it was found that the flagellates grew best and multiplied in number in a medium containing the proportion of one part of human blood serum to four parts of Locke's solution. There were limited growth and reproduction in a medium made from one part of either horse or sheep serum to four parts of Locke's or Ringer's solution. But a culture medium containing Ringer's solution did not give as satisfactory results as one containing Locke's solution, and human serum promoted better growth than either the sheep or horse serum.

The human serum<sup>7</sup> comprised the unused portions of the samples sent in to the laboratory for the Wassermann reaction. Both negative and positive sera were used, and they were equally satisfactory.

<sup>7</sup> This serum was received in a sterile condition from the Department of Bacteriology of the Baltimore City Board of Health through the kindness of Dr. William R. Stokes.



The Locke's solution was made up as follows:

Sodium chloride.....	0.9	gm.
Calcium chloride.....	0.024	"
Potassium chloride.....	0.042	"
Sodium bicarbonate.....	0.02	"
Distilled H <sub>2</sub> O.....	100.00	cc.

To this solution 0.25 gm. of dextrose was added, and it was then sterilized. After the sterilization of the Locke's solution the human serum was mixed with it in the proportion given above, care being taken to prevent any contamination of the resulting medium. The culture medium was distributed in 5 cc. amounts in small test-tubes and these were incubated at 37°C. over night and examined the next morning. The tubes which showed any evidence of bacterial growth were discarded. The medium generally had a clear cream-colored appearance unless blood cells were present in the blood serum, in which case the medium was reddish in color until the blood cells sank to the bottom of the tube. If in the samples of blood a slight amount of hemolysis had taken place, the serum was tinted red by the hemoglobin. Culture medium containing either red blood cells or hemoglobin as the result of the hemolysis of the cells did not prevent the growth and propagation of the flagellates but at times it appeared more favorable for their growth than culture medium devoid of these substances.

The reaction of several batches of the culture medium varied from neutral to 0.4 per cent alkaline to phenolphthalein. A reaction of 0.2 per cent alkaline usually resulted from the mixture of the human blood serum and Locke's solution.

*Technique.*—It was found advisable to wash a small portion of the stool containing the flagellates before inoculating the culture medium, in order to get rid of a superabundance of bacteria, fecal debris, and products of chemical decomposition which in the beginning might prove inhibitory to the growth of the organisms in artificial medium. Accordingly a loop of fecal material containing numerous flagellates was washed by stirring it in with about 2 cc. of warm sterile normal saline solution and then taking a couple of drops of this suspension containing the flagellates and mixing it with another small amount of normal saline solution. This procedure was followed a third time

if it was found that the second resulting suspension was very turbid from the presence of much fecal detritus and numerous bacteria. A couple of drops from each of the above suspensions were inoculated into each of several tubes of culture medium and incubated at 37°C.

On the following day the tubes were examined. Some showed an absence of flagellates; others showed the presence of *Chilomastix* but in small numbers. Transplants were made from the positive cultures by transposing several loops of medium containing the flagellates. On the 2nd day after the original inoculation the positive tubes showed an increase in the number of organisms. Their number was increased even more the following day, at which time more transplants were made. The first inoculation was begun on January 27, 1920, and transplants were made at various intervals. The flagellates were kept in this artificial medium from the above date to June 15, 1920, when an accident to the heating device of the incubator caused the temperature to rise to 80°C., which killed the flagellates.

Other attempts to grow *Chilomastix* taken from the feces in the Locke-human serum medium have proved successful and have demonstrated the favorableness of this artificial medium as a habitat for these parasitic flagellates of man.

#### *Growth in Cultures.*

*Period of Lag.*—The life of a culture, as denoted by the presence of living flagellates, varied in length from 2 to 10 days. The average length of life for each culture was about 6 to 8 days.

The transplants from old to new cultures were usually made in the morning. Such inoculations into new cultures always carried over numerous flagellates and bacteria from the old culture. Little or no growth was noticeable during the early hours of incubation of the new cultures. A period of lag, similar, perhaps, except in length, to the lag phase in fresh cultures of bacteria, apparently characterized the growth of the flagellates during the early hours of the life of a culture. The evidence for a lag phase in the growth of these protozoa in cultures was obtained from the observation of cultures at frequent intervals during the first 22 hours of incubation.

A culture of *Chilomastix* which was transplanted at 9 a.m. and kept in the incubator at 37°C. was observed hourly throughout the same day and night up to 7 a.m. of the following day. Likewise vaseline preparations on depression glass slides were inoculated at the same time as the above culture and observed at the same intervals. Several loops of the culture were withdrawn for observation at each interval of an hour. The vaseline preparations permitted observation of their entire contents, since these preparations had been made upon glass slides which were easily manipulated upon the stage of the microscope.

The first signs of dividing organisms were noticed at 10 p.m. and the flagellates were scarce in number. At midnight, 15 hours after inoculation, the dividing forms were encountered more frequently, and dividing flagellates were in evidence at all subsequent examinations. The number of dividing forms seen during any one observation never exceeded six, but in view of the small volume of several loops of medium taken for each observation, the rate of reproduction was high. The period of lag, in the case of this particular culture, was from 9 a.m. until 10 p.m., or 13 hours in length. During this period of lag it is more than probable that multiplication of the organisms was going on, but the organisms were so few in number that they were missed in the small sample of medium taken for observation. In view of this circumstance it cannot be definitely concluded that the period of lag is 13 hours in length, for with more experiments it may be found to vary considerably.

*Presence of Bacteria.*—During the 1st day in the life of a culture, much gas was produced by the bacteria. A slight scum appeared on the surface of the medium and small bacterial clumps were present at the bottom of the culture. The scum became thicker and the precipitation of bacteria forming clumps became greater during the subsequent days of growth in the cultures.

Most of the bacteria present in the cultures were identified and were chiefly members of the colon-typhoid group. The bacteria identified were *Bacillus coli communis*, *Bacillus coli communior*, *Bacterium aerogenes*, *Bacillus alkaligenes*, and *Bacillus proteus*. *Bacillus subtilis* was found in a few cultures; whether or not it promoted or inhibited the growth of the protozoa was not determined. It

was not present in early cultures and no transplants were made from cultures containing this bacillus.

At the death of a culture, when all the flagellates had disappeared, the reaction of the medium had been changed by the activities of the bacteria. The reaction at the end was always more strongly alkaline. One would expect that the presence of *Bacillus coli*, *Bacterium aerogenes*, and *Bacillus proteus* would produce a sufficient quantity of acid by the fermentation of the dextrose in the medium to turn the alkaline reaction of the medium in the beginning, to acid, 8 days later. The gas present in the cultures during the first few days after inoculation was the result of the fermentative action of *Bacillus coli*, *Bacillus proteus*, and *Bacterium aerogenes*. During this time it is probable, for it was not determined, that the reaction might have been slightly acid, but the effect in such a case was only temporary as the reaction had become alkaline in the end. The increase in alkalinity might have been due to the presence of *Bacillus alkaligenes*, for it was found that this organism was the most predominant of all the bacteria, as evidenced from the number of colonies on an agar plate planted from a culture in which all the flagellates had disappeared.

Most of the flagellates were found feeding upon the bacterial clumps at the bottom of the culture. They were so numerous on the 3rd or 4th day after inoculation of the cultures that they often appeared as a compact mass, with their bodies touching each other and showing little or no progressive locomotion. The presence of most of the flagellates at the bottom of the culture would indicate their preference for a medium in which the oxygen content was somewhat reduced.

*Size of Flagellates.*—The size of the flagellates in the cultures ranged from 3 to 18 microns in length and from 2 to 9 microns in width. During the first 3 or 4 days when the flagellates were numerous, there was a great number of small forms measuring from 3 to 8 microns in length. The small size of these forms was undoubtedly due to the rapid rate of reproduction which took place during the 1st day especially, and later, during the 2nd, 3rd, and 4th days. This rapid proliferation on the part of the flagellates did not permit of the organisms reaching their full growth previous to another division. Many of the small forms were probably the products of multiple fission. Near

the end of the life of the culture when the number of flagellates was small, the organisms were apparently normal in size; *i.e.*, about 8 to 14 microns in length.

The difference in the average size of the flagellates in the cultures and those obtained directly from the feces was due apparently to a more rapid rate of multiplication in the culture than in the intestine of man. The factors which might have caused this great rate of reproduction in the cultures had perhaps a better opportunity of influencing the growth of the protozoa in the test-tube than in the intestine of man, since they were concentrated into a smaller area. And furthermore, they accumulated more rapidly in the cultures than in the human intestine where digestion was accomplishing changes in the intestinal medium of the organisms.

*Cysts.*—Thus far, no cysts have been found in any of the cultures. Whether encystment was inhibited by the lack or presence of certain substances in the medium was not ascertained. Skatole has been known to cause encystment of amebæ, but as yet no experiments have been made with the use of this product of protein decomposition to induce the encystment of *Chilomastix*.

*Rapidity of Growth.*—A detailed study made on five different cultures of *Chilomastix* furnished data as regards the growth and multiplication of the flagellates. The activities of the flagellates in these cultures were not unlike those in all the cultures made of *Chilomastix*.

The number of flagellates per cubic centimeter of culture medium was computed for each day during the lifetime of the five cultures. The method of estimating the number of flagellates per cubic centimeter was as follows: Each culture was shaken vigorously for 1 minute previous to examination in order to distribute the organisms uniformly throughout the medium. Only a sufficient amount of the medium containing the flagellates was placed in the counting chamber of a hemacytometer to prevent an overflow into the moats of the chamber. Immediately before placing the flagellates in the counting chamber, a minute drop of saturated corrosive sublimate solution was placed on the floor of the counting chamber and mixed with the culture medium as both were drawn into the chamber by capillary attraction. The drop of saturated corrosive sublimate solution was used to kill the flagellates, thus preventing their moving

about over the ruled area and avoiding more than one count. This minute drop of corrosive sublimate solution increased the percentage of error to some extent, yet the total count of organisms per cubic centimeter as determined by this method can be said to be fairly representative of the actual number. All the flagellates were counted which were found lying within the confines of the total ruled area of the counting chamber and by simple calculation the number of flagellates in a cubic centimeter was computed.

Each of the five cultures containing 5 cc. of medium with a reaction of 0.1 per cent alkaline to phenolphthalein was inoculated on May 19, 1920. Each culture was inoculated with three loops of medium from another culture having about 200,000 organisms per cubic centimeter. In a representative loopful, about 250 flagellates were counted, hence it follows that each culture received about 750 organisms, or about 150 flagellates for each cubic centimeter of the new culture.

The number of organisms per cubic centimeter from day to day for all five cultures is given in Table I. The per cent increase in the number of flagellates is also given for each day.

On the following day, May 20, there was evidence of the great fertility of these flagellates in the great increase in their number. Culture 3, which contained such a scattering of organisms that they were too scarce to be counted with the hemacytometer, was an exception; but it behaved similarly on the following day. This latter culture showed no apparent increase in the number of flagellates and in all probability contained few more than the original number inoculated. Proliferation had been inhibited or delayed for a period of 1 day. The remarkable fecundity of these flagellates is surprising when one notices in Culture 2 an increase in the number of flagellates from 150 per cubic centimeter to 26,666 per cubic centimeter, an actual increase over the number of May 19 of 17,677.3 per cent. Cultures 1, 4, and 5 also showed remarkable increases in the number of flagellates, Culture 4 being the lowest with an increase of 5,405 flagellates, or 3,603.3 per cent, over the number of the day before. As has been said, the greatest multiplication takes place during the 1st day.

On the next day, May 21, the per cent of increase in the number of flagellates had fallen for Cultures 1, 2, 4, and 5, although the per cent increase for Culture 4 was about the same as that of May 20. Culture 3 showed a large increase from about 150 to 11,111 flagellates per cubic centimeter, an increase of 7,307.3 per cent. Culture 5 had the largest number of organisms per cubic centimeter, which was about 277,777. Culture 2 followed closely.

TABLE I.

Date.	Culture No									
	1		2		3		4		5	
	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.	No. of flagellates per cc.	Per cent.
1920										
May 20	15,555	<10,270.0	26,666	<17,677.3	Few.	<7,307.3	5,555	<3,603.3	20,000	<13,233.3
" 21	150,000	<864.3	253,000	<848.7	11,111	<2,540.0	204,444	<3,580.3	277,777	<1,288.8
" 22	255,555	<70.3	702,222	<177.5	293,333	<25.4	437,777	<114.1	725,555	<161.2
" 23	280,000	<9.5	300,000	>57.2	368,000	<25.4	524,000	<19.7	365,555	>49.6
" 24	232,222	>17.0	208,888	>30.3	357,777	>2.7	472,222	>9.8	401,111	<9.7
" 25	216,666	>6.6	153,333	>26.5	255,555	>28.5	327,777	>30.5	Dead.	
" 26	24,444	>88.7	Dead.		23,333	>90.8	Dead.			
" 27	Dead.				Dead.					

Cultures inoculated May 19, 1920. Number of flagellates per cubic centimeter and per cent increase recorded for each day. < indicates increase; >, decrease.

On May 22, the per cent increase in the number of organisms dropped again for all the cultures including Culture 3. 3 days after inoculation the maximum number of flagellates per cubic centimeter was reached in Cultures 2 and 5 which was 702,222 and 725,555 respectively. Culture 3 showed a much larger number than the day before, increasing from 11,111 to 293,333 flagellates per cubic centimeter, an increase of 2,540 per cent.

On May 23, Cultures 2 and 5, having reached the maximum number of flagellates per cubic centimeter on the previous day, now showed a marked decrease per cubic centimeter of 402,222 flagellates (57.2 per cent) for Culture 2, and 360,000 flagellates (49.6 per cent) for Culture 5. 4 days after inoculation, Cultures 1, 3, and 4 contained the largest number of flagellates per cubic centimeter, although the number of flagellates for each culture was considerably less than the number attained in Cultures 2 and 5 on the day before. Also the per cent increase in the number of organisms for Cultures 1, 3, and 4 was comparatively low.

On the next day, May 24, Cultures 1, 3, and 4 first began to show a decrease in the number of flagellates, but the decrease, ranging from 2.7 to 17 per cent was much smaller than the initial per cent decrease of Cultures 2 and 5 on the previous day. And on this day, Culture 2 continued to decline in its number of flagellates but Culture 5 showed evidence of some increase, for it gained from 365,555 to 401,111 flagellates per cubic centimeter. Nevertheless, it still showed a 44.7 per cent reduction from the maximum number of organisms of May 22.

On May 25, all the cultures showed a greater falling off in the number of flagellates from the day before, and Culture 5 no longer contained flagellates. A drop of 401,111 flagellates per cubic centimeter to none at all had occurred in the 24 hour interval between examinations.

On May 26, Cultures 2 and 4 were also found to contain no flagellates, while Cultures 1 and 3 had suffered a tremendous loss. The decrease was 88.7 and 90.8 per cent, respectively. These large decreases in the number of flagellates and the per cent were forewarning that the organisms would not be found alive much longer, and on the next day, May 27, all had disappeared.

From the study of these five cultures, it was seen, as it had been noticed in all previous cultures, that the period of the greatest rate of proliferation took place during the 1st day and that the greatest number of flagellates was to be found in the cultures on the 3rd or 4th day. Rarely a second phase of multiplication occurred, and when present it usually came on the 5th day when a marked decrease in the number of organisms had already taken place on the day before. Culture 5 showed evidence of this behavior. The rate of increase in the number of flagellates decreased after the 1st day, becoming less and less up to the 3rd or 4th day. Then a decrease in the number of



flagellates occurred, which ended in their disappearance on the 6th, 7th, or 8th day. The rate of increase and decrease in the number of organisms varied for each culture, although they were somewhat similar, in that one could expect an increase during the early days in the life of a culture and a decrease after the 3rd or 4th day. In some instances the rates in per cent of increase and decrease were similar for different cultures. The death and disappearance of all the organisms on the last day were sudden, thousands of flagellates per cubic centimeter being destroyed within a single day.

*Food.*—The food of *Chilomastix* was comprised chiefly of bacteria although the mineral salts of the Locke's solution, the proteins of the blood serum, and the sugar may also have served to some extent as food and a source of energy. But one cannot observe the flagellates using the latter substances as food as they do the bacteria in the medium. When the bacteria are ingested they may be seen within the food vacuoles of the cytoplasm. The process of ingestion has been described above.

It is probable that the presence of the bacteria whether they were for the purpose of food or not may explain in part some of the more important questions concerned with the life of the flagellates in the cultures.

*Fertility and Death of the Flagellates.*—No definite evidence has been uncovered as yet to explain the great fertility of the flagellates during the 1st day in a new culture but certain theoretical conclusions warrant consideration. It has already been said that a period of lag preceded the period of excessive and rapid multiplication. This lag period, as yet, has not had its length of time determined, although the first dividing flagellates were found 13 hours after the inoculation of the cultures.

In the growth of bacteria a period of lag has been known for some time past. Lately, Salter<sup>8</sup> working with *Bacillus coli communis* showed that the length of this period was from 3 to 8 hours, depending upon the medium used and the age of the original culture from which subcultures were made. It is probable that the period of lag of

<sup>8</sup> Salter, R. C., Observations on the rate of growth of *B. coli*, *J. Infect. Dis.*, 1919, xxiv, 260.

*Bacillus coli communis* is similar in length to the period of lag for other members of the colon-typhoid group in favorable media.

The bacteria present in the cultures of *Chilomastix* belong chiefly to the colon-typhoid group. Since some of these bacteria were used as food, the food supply of the flagellates was scarce during the lag phase of the growth of the bacteria. This scarcity of food supply might have been in part responsible for the period of lag in the growth of the flagellates when little or no multiplication occurred. The bacteria passing through the lag phase then entered upon a period of great multiplication. Within several hours the bacteria increased in number from a few hundred to many million. This great number of bacteria in the cultures furnished adequate food for the flagellates, and it seems only natural that the period of great fertility of the flagellates should now follow in the presence of this food supply.

Chemical decomposition products of bacterial metabolism have been forming in the cultures which have probably altered the culture medium in such a way as to make it most favorable for the growth of the protozoa. Because of these circumstances, it is very likely that the period of great fecundity of the flagellates was dependent to some extent upon the previous period of rapid multiplication of the bacteria, by virtue of which the culture medium was rendered favorable for the growth of the protozoa and an abundant food supply was obtained.

Table I shows that the rate of increase of the flagellates in the cultures began to decline after the 1st day. From the 3rd or 4th day on, the flagellates decreased in number and in most cases all had disappeared from the cultures on the 6th, 7th, or 8th day. This characteristic increase and decrease in the number of flagellates after the 1st day might also have been due to the metabolic relations existing between the protozoa and the bacteria, as appears to have been the case in the explanation of the great fecundity of the flagellates on the 1st day.

The chemical products of decomposition brought about by the metabolic activities of the bacteria accumulated rapidly during the first 24 hours, during which time the rate of multiplication of the bacteria was at its height. The amount of the chemical substances produced within this time was just sufficient to alter the medium

to the point where it was most favorable to the proliferation of the protozoa. Following the period of rapid reproduction of the bacteria, their rate of multiplication was slower and more or less constant; nevertheless, the chemical products were being formed and accumulated, but at a slower rate. Furthermore, mortality among the bacteria occurs at a slow rate in the early life of a culture but increases with the age of the culture. So in the culture of *Chilomastix*, as the death rate of the bacteria increased, the decomposition of their bodies produced other substances in the medium which might have been detrimental to the life of the flagellates.

Just as a minimum amount of these products of decomposition might have served to make the culture medium favorable for the rapid multiplication of the flagellates during the 1st day, so, through their accumulation from day to day, an excess of these substances may have become poisonous and thus inhibited the proliferation of the flagellates at a rapid rate after the 1st day. If this were the case, the continued accumulation of these now poisonous products caused a decrease in the number of the protozoa after the 3rd day and ultimately brought about the death and complete disappearance of the flagellates from the culture.

Some experiments have been performed upon amebæ, which, with the use of certain products of protein decomposition, resulted in an increased rate of cell division. The results of these experiments appear to lend evidence in support of the above theory that the chemical products of protein decomposition promoted the proliferation of the flagellates when present in small amounts, and that they became poisonous when present in larger amounts. The action of these substances as found in the experiments upon the ameba has well defined limits within which cell division is induced at a rapid rate, and beyond which death occurs.

The researches of Fantham<sup>9</sup> on *Entamæba coli*<sup>10</sup> showed that tyrosine, an aromatic compound and a product of protein putrefaction, when present to the

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<sup>9</sup> Fantham, H. B., On the amœbæ parasitic in the human intestine, with remarks on the life-cycle of *Entamæba coli* in cultures, *Ann. Trop. Med. and Parasit.*, 1919, v, 111.

<sup>10</sup> Dobell (Dobell, C., The amœbæ living in man, London and New York, 1919) believed that the organism which Fantham cultured was not the parasitic ameba, *Entamæba coli*, but a free living species.

extent of 0.2 per cent in the culture medium, had the effect of shortening the life cycle, increasing the number of generations in any one culture, and increasing the frequency of binary fission.

Similarly the work of Cropper and Drew<sup>11</sup> on the cysts of the free living ameba, *Acanthamoeba*, showed that tyrosine (3 per cent) caused excystation in 24 hours; the free forms became larger, and among them were many multinucleate types. Choline, a member of the amine group and a product of the decomposition of amino-acids, showed even stronger powers in inducing cell division than the tyrosine. In one experiment two culture slides, A and B, contained equal volumes of 0.2 per cent choline in distilled water and distilled water, respectively. Upon A were 34 amebæ and on B, 26 amebæ. After 3 days the number of amebæ on A had increased from 34 to 207 and on B from 26 to 88. These investigators concluded that the substances "undoubtedly in certain strengths cause great proliferation of amebæ," but also that when a certain strength of these substances is exceeded "the amebæ are poisoned and multiplication is retarded."

In the study of the five cultures given in Table I, the reaction of the medium was changed from 0.1 per cent alkaline to phenolphthalein in the beginning, to 0.3 per cent alkaline at the end when all the flagellates had disappeared. The question arises as to whether or not the change in the reaction of the medium was responsible for the death of the flagellates. The change in the reaction may have been a contributing factor in the death of the flagellates, but at this time it does not seem fair to attach too much importance to this factor, because the flagellates thrived very well in some batches of medium with an initial reaction of 0.3 per cent alkaline to phenolphthalein which became more alkaline at the death of the culture. In view of this evidence it seems more probable that the death of the flagellates was brought about through the poisonous action of the chemical decomposition products of bacterial metabolism and not as a result of a change of the reaction of the culture medium.

In view of this theory, the persistent character of infections of man by *Chilomastix* may be explained in part. The accumulation of the products of protein decomposition does not normally occur in the intestine of man since the act of defecation carries them to the outside. The disappearance of the flagellates from the stools for short or long intervals might be explained by the death of numerous flagellates

<sup>11</sup> Cropper, J. W., and Drew, A. H., *Researches into induced cell-reproduction in amebæ*, London, 1914.

through the toxicity of the substances of protein decomposition, temporarily accumulated, which in turn have altered the digestive tract to such an extent as to make it an unfavorable habitat. The removal of the substances would bring about a period of great proliferation which would be indicated by the presence of the flagellates in the stools again.

### *Reproduction in Cultures.*

No detailed account of the methods of reproduction of *Chilomastix mesnili* will be given in this paper because of the inability thus far to study a sufficient number of stained preparations. It is hoped that at a later date this may prove possible. The observations to be recorded at this time were made chiefly upon living organisms, as the dividing forms were encountered during the examination of the cultures.

*Binary Fission.*—Binary fission was the type of cell division most often encountered, and in all probability it was the chief method of multiplication in the cultures after the 1st day. In the early stages of binary fission, the dividing flagellate was somewhat ovoid in shape, devoid of bacteria in most cases, and marked by the absence of a posterior protuberance. The first stage observed indicated an advanced period of division, for two nuclei were seen as bright refractile areas and a cytostome was present on either side. It could not be definitely determined whether a double set of anterior flagella was present or not, but there appeared to be a cytostomal flagellum for each oral pouch.

As the plasmotomy of the cytoplasm progressed, it was noticed that the plane of division was median and longitudinal. The separation of the two daughter flagellates began by a cleavage of the cytoplasm which proceeded anteroposteriorly. The division was apparently equal for both resulting organisms. The further stages in plasmotomy were aided considerably by the flagellar action of the organisms. The resulting daughter organisms tugged in opposite directions and the terrific lashing activity of the flagella no doubt served to separate the individuals. Just previous to their final separation, the two daughter flagellates were bound together by a fine, terminal strand of cytoplasm composed apparently of only the

hyaline periplast. The breaking of this strand set the organisms at liberty, while the resulting portions of the strand became the posterior spine for each flagellate. In one instance, approximately 35 minutes elapsed between the stage wherein plasmotomy had just begun to the final separation of the daughter flagellates.

From some stained preparations, there was noticed the formation of chromosomes, whose number appeared to be four, also the division of the centrosomes with the formation of a paradesmose. The paradesmose increased in length during the separation of the daughter nuclei. No study has been made of the different phases of mitosis, but the observations which have been made thus far have been in part recorded by Chalmers and Pekkola.<sup>1</sup>

*Multiple Fission.*—Evidence of multiple fission was also noticed in the cultures of *Chilomastix*. It is probable that this method of reproduction was the one most often resorted to during the 1st day, when the rate of multiplication was at its highest. The only flagellates apparently undergoing multiple fission were found during the first 22 hours in a culture which had been observed at intervals of an hour throughout 1 day and night until the following morning. Stained preparations were made, which revealed a few forms possessing four incomplete sets of organella.

In the living forms undergoing multiple fission, as in the case of binary fission, no posterior protuberance was visible. The body of the flagellate was large and spherical, in one instance measuring 19 microns in diameter. These forms did not appear to contain any bacteria, but the cytoplasm was more or less uniform in its granular constitution. The organism exhibited distinct ameboid movements by the bulging out and subsequent withdrawal of considerable areas of its body. The parts of the body which were so pushed out were usually equipped with flagella and a cytostome.

The body of the flagellate was a somatella comprising four zooids. The somatella possessed four distinct cytostomes located in different sectors of the body. Each cytostome had a cytostomal flagellum and nearby there appeared to be a single anterior flagellum. The nuclei were not seen in the living forms, but in the stained preparations the forms undergoing multiple fission possessed four nuclei. The nuclei were located in different sectors also, and near the cytostome of the respective sector.

*Conjugation.*—Whether or not other observations made upon *Chilomastix* in the cultures are to be interpreted as probable signs of conjugation can only be determined by further investigation. It seems advisable to record the observations of this nature made thus far.

It was noticed in the cultures, chiefly in those which were only 1 to 3 days old, that some of the flagellates were joined together in pairs. But while in this position, each flagellate was very actively beating with its flagella, and the manner of their union was such as even to permit the reversal of their respective positions. In the majority of these cases the common position of their bodies was such that their anterior ends were pointed toward the same direction, and when reversed the anterior end of each flagellate would point in the opposite direction. This reversal of direction occurred for only a moment, after which the original position was assumed again. Such forms were observed for a long time, but they were never seen to separate.

At present in the absence of more evidence from stained preparations one cannot attribute any syngamic significance to these double forms, since no actual transfer of nuclear substance was noticed. The two bodies of the flagellates in union did not apparently fuse together, for a line marking the confines of the cytoplasm of the organisms was always visible at the point of junction.

It must be stated, too, that clumping of the flagellates also took place at the edge of the cover-slip preparation, where the oxygen was more abundant. It does not seem, however, that the union of flagellates in pairs as described above was of the same nature as the clumping of the organisms in aggregates of a dozen and more in the area of the greatest abundance of oxygen. The paired forms were often seen after a sample had been taken directly from the cultures, at which time no clumping of the flagellates had occurred at the edge of the cover-slip.

#### SUMMARY.

1. Cases of flagellate infection of man by *Chilomastix mesnili* have been reported from nearly every locality in the world. They are fairly abundant in the United States and are often confused with cases of infection by *Trichomonas intestinalis*.

2. The shape of *Chilomastix* is pyriform. The body contains the following structures: nucleus, centrosome, primary, secondary, and tertiary blepharoplasts, parabasal, parastyle, peristomal fiber, ventral cytostome, cytostomal flagellum, three anterior flagella, food vacuoles with bacterial inclusions, and a posterior spine-like process, the hold-fast organ.

3. The twisted shape in some flagellates in this case of infection is a temporary condition. It was seen in flagellates taken from the feces of the patient on only two occasions and was never observed among the cultured forms.

4. The cysts of *Chilomastix* are lemon-shaped and occurred irregularly in the stools of the patient. They have not been encountered thus far in the cultures.

5. *Chilomastix mesnili* was cultured continuously from January 27 to June 15, 1920, in an artificial medium composed of one part of human serum and four parts of Locke's solution with the addition of a small amount of dextrose.

6. Cultures sometimes remained viable for a period of 1 to 10 days, but generally for a period of 1 to 8 days.

7. The flagellates in the cultures increased in number during the 1st, 2nd, 3rd, and often the 4th days, after which there was a decrease until all had disappeared.

8. A period of lag, probably 13 hours in length, and followed by a period of great fecundity, characterized the growth of the flagellates during the 1st day in the cultures.

9. The number of flagellates in the cultures varied considerably and often reached 700,000 per cubic centimeter.

10. The bacteria served in part as the food of the flagellates. The bacterial organisms identified in the cultures were *Bacillus coli communis*, *Bacillus coli communior*, *Bacterium aerogenes*, *Bacillus alkali-genes*, and *Bacillus proteus*. The proteins of the blood serum, the mineral salts, and the dextrose in the medium may also have proved a source of food for the protozoa.

11. The products of bacterial metabolism, resulting from the growth of the bacteria, their fermentative activity, and their death may explain the rate of growth, reproduction, and death of the flagellates in the cultures. A certain amount of the products of decomposition



together with the presence of the bacterial food supply may explain the rapid proliferation of the flagellates during the 1st day and the slower rate up to the 3rd or 4th day. An excess of these products may have inhibited the growth and multiplication of the protozoa, especially after the 3rd or 4th day, and ultimately may have brought about their death and disappearance from the cultures.

12. The initial alkalinity of the medium increased at the death of the culture. It is not believed that the change in alkalinity proved lethal to the protozoa.

13. Binary fission was often observed. The plane of division was median and longitudinal. A paradesmose was formed.

14. Multiple fission also occurred. The flagellate body, ameboid in movement, was a somatella comprised of four zooids. It contained four cytostomes, each with a cytostomal flagellum. The number of anterior flagella was not complete for each zooid.

15. Flagellates, joined in pairs, were often seen during the first 3 days in the cultures. They may represent conjugating forms, but as yet no evidence of the exchange of nuclear substance has been seen.



## MECHANISM OF UREA EXCRETION.

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PLATES 8 AND 9.

(Received for publication, August 2, 1920.)

Since the time of modern physiological investigation, two theories have dominated our conception of the mechanism of renal excretion. Bowman in 1842, basing his deductions on the anatomical structure of the renal unit, suggested that the glomerulus furnished the water of the urine, and that the solid substances were added to it by the activity of the renal cells which line the tubule. The experimental evidence for this view was furnished by Heidenhain<sup>1</sup> and his pupils in a long series of investigations.

2 years later, Ludwig<sup>2</sup> advanced his theory that all the urinary constituents were derived by filtration through the glomerulus, the ultimate concentration of the urine being arrived at by a process of absorption of water by the renal tubule. Both processes were held to be purely physical.

The various modifications which have been offered by the pupils of these two schools and by other observers are innumerable. The most recent concept is that of Cushny,<sup>3</sup> and is termed by him the modern theory. While neither of the original theories can explain the mass of physiological and morphological data that has accumulated since their origin, the modern theory covers in a much more satisfactory manner our present knowledge.

According to Cushny, the formation of the urine may be explained by two processes: first, a purely physical filtration through the glomerulus of all the constituents of the plasma except the colloids; and, second, a resorption from this filtrate by the vital activity of the epithelium as it passes down the tubule. The former process furnishes the urinary constituents, the latter modifies their amounts so that they correspond to those of the completed urine.

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<sup>1</sup> Heidenhain, R., in Hermann, L., *Handbuch der Physiologie*, Leipsic, 1883, v, 279.

<sup>2</sup> Ludwig, C., *Lehrbuch der Physiologie des Menschen*, Leipsic and Heidelberg, 1861, ii, 373.

<sup>3</sup> Cushny, A. R., *The secretion of the urine*, London and New York, 1917.

Water is obviously the substance which will be handled in greatest amount by both filtration and absorption, and the conception of such a double process with this substance furnishes little difficulty. A complication arises, however, when the solid constituents are considered, as a uniform rate of absorption will not explain the varying levels of concentration that the different solids show in the completed urine. These are therefore divided into "threshold" and "non-threshold" substances. The former appear in the urine only as they exceed a certain value below which they are completely absorbed. Sugar and the chlorides are examples. The non-threshold substances, on the other hand, are found in the urine in direct proportion to their absolute amounts in the plasma, as they are not absorbed. Of these, urea and sulfates are the most important. The urine therefore contains all the urea of the glomerular filtrate and none of the sugar.

TABLE I.

*Amount of Filtrate and Absorbed Fluid Required to Form 1 Liter of Urine.*

Blood urea 70 mg. per 100 cc.; urine urea 5.5 per cent; urine 1,000 cc.

	Urea.	Water.
	<i>gm.</i>	<i>cc.</i>
Blood.....	0.07	100
Glomerular filtrate.....	55.0	78,500
Amount absorbed by tubule.....	0.0	77,500
Urine.....	55.0	1,000

Table I shows the amount of filtrate and absorbed fluid which would be required to form 1 liter of urine in an actually observed case of high urea excretion. The figures in the table hold only in case the entire urea content of the blood is available for filtration. In a recent article Cushny<sup>4</sup> claims that as the urea is distributed in about equal amount between the plasma and the corpuscles, only that half free in the plasma can pass through the glomerular filter. In this case, to obtain the 55 gm. found in the urine, twice as much filtrate would be needed as is shown in the table; 157,000 cc. of filtrate would be formed, of which 156,000 cc. would be absorbed.

Such a mechanism, though perhaps indirect, will nevertheless account for those alterations which would be required to convert the

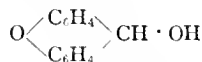
<sup>4</sup> Cushny, A. R., *J. Physiol.*, 1919-20, liii, 391.

blood plasma to urine without the intervention of a secretory factor. The completeness with which the various experimental and clinical facts are covered by this theory is well shown by Cushny.<sup>3</sup> Even changes in the mitochondria and the appearance of dyes in the tubule cells, which Heidenhain considers morphological evidence of a secretory process, may be equally well accounted for by the assumption of a vital absorption from the lumen of the tubule. This also applies to the demonstration of uric acid in the tubule cells, as this substance is one of the threshold group, and, according to the modern theory, is absorbed from the lumen of the tubule during concentration of the urine.

Urea, however, is a non-threshold substance and, if demonstrated in the renal cells, cannot be explained by a process of absorption from the lumen. The concentration of urea in the glomerular filtrate must be raised to that of the urine, and this could be done slowly, or not at all, if urea was absorbed along with the water.

Urea has been demonstrated in the proximal convoluted tubule by Leschke<sup>5</sup> and his experiments have been confirmed by the writer.<sup>6</sup> The method, which depends on the formation of an insoluble compound between urea and mercuric nitrate, may be criticized, as the resulting reaction is not so distinctive as might be desired. The protoplasm of all cells reacts more or less, though always less than that of cells of the proximal convoluted tubules. Whether this is due to the delicacy of the reaction, for Marshall and Davis<sup>7</sup> have shown the widespread distribution of urea in all tissues, or to a lack of specificity, is impossible to say. The quantitative variation in the degree of the reaction in the cells of the proximal convoluted tubule depending on the concentration of urea in the secreted urine has been taken as evidence of its specificity.<sup>6</sup>

Subsequently a new qualitative reagent for urea has been described by Fosse.<sup>8</sup> This is xanthidrol



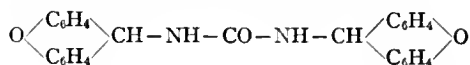
<sup>5</sup> Leschke, E., *Z. klin. Med.*, 1915, lxxxi, 14.

<sup>6</sup> Oliver, J., *J. Exp. Med.*, 1916, xxiii, 301.

<sup>7</sup> Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

<sup>8</sup> Fosse, R., *Bull. sc. pharmacol.*, 1914, xxi, 74, 502.

which forms with urea a characteristic crystalline product



The crystals are insoluble in acetic acid and differ in this way from the compounds which xanthydrol forms with other substances.

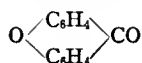
Two attempts have been made to demonstrate urea in the tissues with this reagent. Policard<sup>9</sup> was unable to find the crystals in any of the injected kidneys, though they were present in the lumina of the collecting tubules. He therefore concluded that urea does not exist in a free form in the kidney cells, but that it is bound in some intimate combination with the protoplasm. Chevallier and Chabanier,<sup>10</sup> however, describe the typical crystals of the urea compound in the cells of the convoluted tubules, in all the vessels of the kidney, and in the lumina of the ducts of Bellini.

The importance of these findings with regard to the mechanism of urea excretion, as well as the need for the accurate localization of the urea in the various parts of the kidney, is obvious. For these reasons the experiments have been repeated with certain modifications.

#### *Technique.*

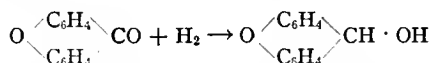
*Reagents.*—As it is impossible to obtain xanthydrol in the market, it was prepared in the following manner.

100 gm. of salol are heated to boiling in a distillation flask and the first fluid fraction, consisting largely of phenol, of 35 to 40 gm., is discarded. The remaining fraction, xanthone,



comes over and condenses in the form of long needles. These are collected and heated with NaOH, washed free of alkali, and purified by recrystallization from alcohol.<sup>11</sup> The xanthone gives a bright yellow color with a light blue fluorescence if treated with concentrated H<sub>2</sub>SO<sub>4</sub>.

The next step consists in the reduction of xanthone to xanthydrol.



<sup>9</sup> Policard, A., *Compt. rend. Soc. biol.*, 1915, lxxviii, 32.

<sup>10</sup> Chevallier, P., and Chabanier, H., *Compt. rend. Soc. biol.*, 1915, lxxviii, 689.

<sup>11</sup> Vanino, L., *Handbuch der präparativen Chemie*, Stuttgart, 1914, ii, 512.

This is accomplished by boiling in a reflux condenser 10 gm. of xanthone, 40 gm. of NaOH, and 400 cc. of alcohol, adding from time to time a pinch of zinc dust so that there is always a small amount present. The process is continued for from 6 to 8 hours, and the fluid then poured into cold water. The xanthydrol comes down in the form of fine crystals which are washed and dissolved in boiling alcohol and recrystallized by pouring again into an excess of water.<sup>12</sup> The xanthydrol gives with concentrated  $\text{H}_2\text{SO}_4$  a bright yellow color with a light green fluorescence.

The mixture used for the injection of the kidneys was made up fresh for each experiment, as follows: 2 gm. of xanthydrol were triturated with 10 to 15 cc. of methyl alcohol, and 20 cc. of glacial acetic acid were added. The turbid fluid was filtered and was then a clear light yellow. If added to water, the xanthydrol comes out of solution, and the same precipitation occurs to some degree in the tissues. These crystals, however, are soluble in alcohol, and their disappearance may be easily followed as the specimen passes through the alcohols during embedding and staining.

*Animals Used.*—Rats were used for the experiments as their kidneys require less reagent than those of larger animals. In order to get a satisfactory concentration of urea in the urine, some animals were either fed a mixture of lard and corn meal containing urea, or a dilute solution of urea was injected into the peritoneal cavity. Others were injected after having lived on a normal diet. The first procedure was preferred as more nearly approaching physiological conditions. The animal was killed, the thorax quickly opened, and the reagent injected by way of the aorta. The injection was continued until the kidney was completely fixed. Thin slices were made and placed overnight in 95 per cent alcohol, the tissue was embedded in paraffin, and sections were stained with hematoxylin.

#### EXPERIMENTAL.

If a rat whose kidney is excreting urine high in urea concentration is injected with the xanthydrol reagent, the organ swells somewhat and turns a light opaque yellow. On the cut surface one can see

<sup>12</sup> Meyer, R., and Saul, E., *Ber. chem. Ges.*, 1893, xxvi, 1276.

with a hand lens that this discoloration is due to the presence of innumerable minute crystals scattered throughout the cortex and appearing in the medulla in long thin stripes.

Microscopic examination of the sections shows the exact location of these crystals. They lie in three places: in the blood vessels, in the cells of certain tubules, and in the lumina of the tubules (Figs. 1 to 5).

#### *The Intravascular Crystals.*

In all parts of the kidney and in the surrounding tissues (perirenal fat), all blood vessels contain typical crystals (Figs. 1, 3, and 5). These are in the form of fine, pointed needles, varying in size from 4 to 50  $\mu$  in length, lying either singly or arranged in the form of rosettes. On account of the arrangement of the vessels, the crystals appear in the medulla in long rows between the straight tubules, while in the tortuous capillaries of the cortex they are more irregularly scattered. They are also seen in the capillaries of the glomerular loop (Fig. 1).

The number of crystals in the individual cross-sections varies greatly; some are empty, others completely filled. This irregular distribution is evidently due to the current of the injection fluid which carries the crystals with it.

#### *The Intracellular Crystals.*

Crystals contained in cells are found only in the cortex, where the thick epithelium of the proximal convoluted tubule is filled with them (Fig. 1). They lie in all parts of the cell, near the membrana propria, in the region of the nucleus, or just beneath the brush border (Fig. 2). They are smaller than those seen in the blood, and when arranged in rosettes are more or less deformed. Their characteristic structure is nevertheless evident.

The number of crystals in the tubule cells gradually decreases so that the terminal spiral portions of the proximal tubule show definitely fewer crystals than the cross-sections which lie in the proximity of the glomerulus.

The other divisions of the renal tubule practically never show intracellular crystals. This contrast is not so evident in the cortex, where the great majority of the cross-sections are of the proximal convoluted



tubule, but at the junction of the outer and inner stripes of the outer zone of the medulla it is striking. Here the large spiral terminal portions of the proximal convoluted tubules, situated in the outer stripe, end suddenly to form the line of demarcation from the inner stripe, which contains the broad ascending limbs of Henle's loop. The former contain the crystals in moderate number, while the latter do not show them (Fig. 3).

#### *The Crystals in the Lumen of the Tubule.*

The lumen of the entire renal unit, from the capsular space to the large ducts of Bellini, contain the crystals, which resemble in their long needle shape those seen in the blood. The increase in number and in the size of the rosettes toward the end of the tubule is striking.

In Bowman's space the crystals are comparatively small and infrequent (Fig. 1). Beginning in the lumen of the proximal convoluted tubule, however, there is a definite increase in their number, which becomes even greater in the narrow descending limb of Henle's loop (Fig. 4). The sections of the small collecting tubules in the cortex show an added increase (Figs. 1 and 3), while in the terminal ducts of Bellini relatively large rosettes are seen (Fig. 5).

As in the case of the blood vessels, not every cross-section of a tubule shows an equal amount of crystals. In those regions where the urine is comparatively dilute, as in the proximal convoluted tubules, many are empty. This may be explained by the fact that a volume of urine produces a relatively much smaller volume of crystalline product, so that as the urea is condensed in crystalline form, it leaves the surrounding areas free. A single rosette of crystals may thus represent the urea from a considerable length of tubule.

#### *Demonstration of Urea Crystals in Other Tissues.*

As a control, the livers of certain of the animals were injected with the reagent. Here the hepatic vessels contained the crystals in the same amount as those of the kidney; the hepatic cells, however, did not show them.

## DISCUSSION.

The interpretation of the above findings is greatly aided by our accurate knowledge of the distribution of urea in the body. It has been shown that all the tissues except the fat contain urea in the same concentration as that found in the blood, the kidney alone exceeding this amount (Marshall and Davis<sup>7</sup>).

From this it follows that the concentration of urea in the cells of the proximal convoluted tubules, which show the crystals of urea, must be higher than that of the blood, as in the liver and the other tubules of the kidney, where the concentration is equal to that of the blood, there is no reaction.

There is therefore a certain threshold below which the reaction does not take place in the protoplasm. The deformity and small size of the intracellular crystals is further evidence of this embarrassment to the reaction. This threshold lies somewhere above the concentration of urea in the blood, and is only reached in the proximal convoluted tubule.

The question now arises as to whether the source of this excess urea is the lumen of the tubule (absorption) or the blood (excretion).

Any theory concerning the mechanism of urea excretion should explain the great rise in urea concentration of the urine as contrasted with that of the blood. By a process of absorption this can only be accomplished if the other constituents of the urine (water) are absorbed and the urea is left behind, and such an assumption is made by the modern theory. Urea would not, therefore, be found in a higher concentration in the cells of the tubule than in the blood.

The excretion of urea, on the other hand, will raise the concentration in the urine, and the high concentration in the cells would be expected. Our demonstration of such a high concentration can therefore only be explained by the assumption of an excretion of urea from the blood into the lumen of the tubules.

That absorption of water without absorption of urea takes place, however, is shown by the increased concentration of urea in the urine as the tubule is descended so that large rosettes of crystals are seen in the ducts of Bellini but none in the epithelium (Fig. 5).

The mechanism of urea excretion may be summarized as follows: Urea passes through the glomerular capsule in the same concentration as that found in the plasma. A certain amount is added to this filtrate by excretion through the cells of the proximal convoluted tubule, and the ultimate concentration is reached by an absorption of water in the remainder of the tubule.

Such a modification will not affect the fundamentals of the modern theory, as Cushny states that "it may be necessary to supplement what I have termed the modern view with an active secretion in the tubules."<sup>13</sup>

#### CONCLUSIONS.

1. Urea is present in the cells of the proximal convoluted tubule in a concentration higher than that of the blood or than that of the cells of any of the other kidney tubules.

2. Such a condition can only be reconciled to an assumption of an active secretion (excretion) on the part of these cells.

3. Urea also passes through the glomerular filter with the other crystalloids of the blood plasma.

4. The final concentration of urea is due to the above mentioned secretion by the proximal convoluted tubule, and to the absorption of water in other parts of the tubule.

#### EXPLANATION OF PLATES.

Drawings made with the aid of a camera lucida. Bausch and Lomb ocular 1, objective  $\frac{1}{2}$ .

#### PLATE 8.

FIG. 1. Rat 1. 2 cc. of 5 per cent urea intraperitoneally; killed 1 hour later. A glomerulus is shown with several sections of the proximal convoluted tubule and one of a collecting tubule. Crystals of urea-xanthydiol are seen in the vessels near the glomerulus, in the loops of the capillary tuft, in the space of Bowman's capsule, and in greater number in the cells of the proximal convoluted tubule. The lumen of the collecting tubule also contains some rosettes of crystals.

FIG. 2. Rat 1. Higher magnification of one of the proximal convoluted tubules. Small rosettes of crystals are seen scattered throughout the epithelial cells. There are some crystals in the vessels. Bausch and Lomb ocular 1, objective  $\frac{1}{2}$ .

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<sup>13</sup> Cushny,<sup>3</sup> p. 52.

FIG. 3. Rat 2. Same as Rat 1. The junction of the inner and outer stripes of the outer zone of the medulla is shown. Above, the larger terminal ends of the proximal convoluted tubules containing crystals in their cells, and in one case a rosette of crystals in the lumen. Below, the smaller ascending limbs of Henle's loop which contain no crystals. Scattered crystals and rosettes are seen in the intertubular capillaries and in the collecting tubule at the left.

#### PLATE 9.

FIG. 4. Rat 3. Fed a mixture of urea (10 per cent), corn meal, and lard for 3 days; killed and injected. The tubules represented were situated in the inner stripe of the outer zone of the medulla. Four ascending limbs of Henle's loop are shown surrounding the loop proper which is formed by the narrow limb of the tubule in this case. Its lumen contains many crystals.

The loop shown in this figure is one of the short type described by Peter,<sup>14</sup> in which the bend lies close to the end of the proximal convoluted tubule. There could have been, therefore, little opportunity for the absorption of water to produce the relatively high concentration of urea as indicated by the mass of crystals.

FIG. 5. Rat 4. Rat on normal diet, no urea given. Margin of the papilla of the medulla. Three large ducts of Bellini are shown, their lumina filled with huge rosettes of crystals. Scattered rosettes are also seen in the pelvis, and a few in the intertubular capillaries.

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<sup>14</sup> Peter, K., Untersuchungen über Bau und Entwicklung der Niere, Jena, 1909.

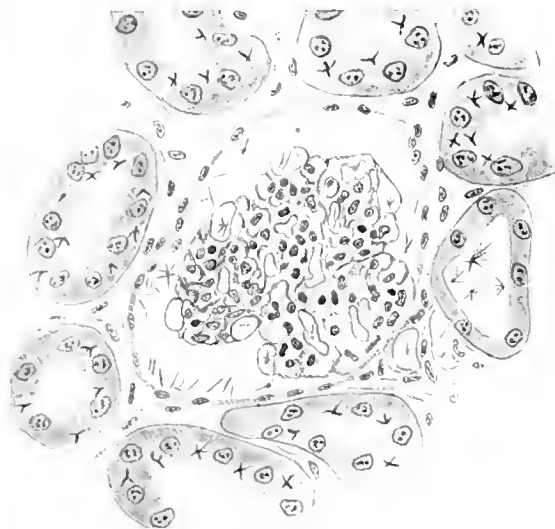


FIG. 1.



FIG. 2.



FIG. 3.

(Oliver: Mechanism of urea excretion.)





FIG. 4.



FIG. 5.

(Oliver: Mechanism of urea excretion.)





## IMMUNOLOGICAL DISTINCTIONS OF ENCEPHALITIS AND POLIOMYELITIS.

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(Received for publication, August 26, 1920.)

In the course of the many discussions of encephalitis lethargica which have followed the pandemic of that unusual disease, the question of its relation to epidemic poliomyelitis has been raised either incidentally or directly. Von Economo,<sup>1</sup> who reported the first Austrian cases, believed that poliomyelitis could be excluded, while Draper,<sup>2</sup> who studied the residual pareses in some of the cases in the English outbreak of 1918, concluded that many of them were true cases of poliomyelitis; however, he called attention to certain phenomena distinctly unusual in experiences with poliomyelitis and left open the question as to whether in the whole group there existed a subsection representing a new disease. Crookshank,<sup>3</sup> in discussing the epidemiology, has expressed the view that poliomyelitis, lethargic encephalitis, and possibly still other paralytic epidemic affections, may be different manifestations of one etiologically simple malady.

There are grave objections to the confusion of the etiology of poliomyelitis and lethargic encephalitis. The divergent clinical manifestations have been more marked as the epidemic outbreak of lethargic encephalitis has endured and become more widely distributed. The great difference in the communicability of this affection as compared with poliomyelitis is another point of capital distinction. Apparently the seasonal variation in the prevalence, namely the occurrence of poliomyelitis in the summer and autumn months and

<sup>1</sup> von Economo, C., *Wien. klin. Woch.*, 1917, xxx, 581.

<sup>2</sup> Draper, G., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects, New Series*, No. 121, 1918, 62.

<sup>3</sup> Crookshank, F. G., *Boston Med. and Surg. J.*, 1920, clxxxii, 34.

of lethargic encephalitis in the winter and spring months is no longer so sharp. Netter<sup>4</sup> has reported summer cases of the latter malady in Paris. Moreover, from information furnished by the Department of Health of the City of New York and the statistics in the *Public Health Reports of the United States Public Health Service* it appears that a similar extension of the period of prevalence is occurring in the United States.

There is one means of distinguishing epidemic poliomyelitis and lethargic encephalitis which has not yet been applied. It relates to the point whether the serum of convalescent cases of lethargic encephalitis can neutralize the virus of poliomyelitis. This fact is readily determined experimentally by the method described by Amoss and Eberson.<sup>5</sup> The principle of the test lies in the power of a neutralizing serum, when administered intraspinally, to prevent the development of poliomyelitis in the monkey following the intravenous injection of a large dose of the virus.

#### EXPERIMENTAL.

The blood serum of four cases of lethargic encephalitis was used in the test, one from a patient convalescent in the 5th week of the disease, the second in the 4th month, the third in the 5th month, and the fourth 15 months after the attack. The tests were controlled by two experiments in which the same procedure was followed, except that one monkey received intraspinal injections of normal human serum and the other intraspinal injections of poliomyelitic serum from a monkey which had had experimental poliomyelitis 9 months before and recovered with residual paralyses.

The virus of poliomyelitis used came from a strain which had been passed from monkey to monkey many times during the past 9 years and which, between passages, had been preserved in 50 per cent glycerol in the ice box. Before starting the tests this virus was passed through three normal monkeys in order to determine its virulence. The certain infecting dose for intracerebral injection<sup>6</sup>

<sup>4</sup> Netter, A., *Bull. et mem. Soc. méd. hôp. Paris*, 1920, xliv, series 3, 1030.

<sup>5</sup> Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1918, xxvii, 309.

<sup>6</sup> All intracerebral inoculations were made under ether anesthesia.

was found to be 0.25 cc. of a Berkefeld filtrate of a 5 per cent suspension of the nervous tissues containing the virus.

The infecting power of the virus when given intravenously is shown in the following preliminary experiment.

A monkey received at 5 p.m. an intraspinal injection of 2 cc. of normal horse serum. The following morning 50 cc. of a 5 per cent suspension of fresh virus were given intravenously. 5 days later the animal was weak in both legs and excited. Both arms were paralyzed on the 6th day and the monkey died on the 7th day.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis were found.

In making suspensions for intravenous injections the tissues used must be fresh. Accordingly, a monkey prostrate from an intracerebral injection of 0.5 cc. of a suspension of the virus 6 days before, was killed with ether and autopsied at once. A 5 per cent suspension of the cord and medulla was prepared for immediate injection.

### *Series 1.*

*Experiment 1.*—*Macacus rhesus* A. Normal human serum control. Mar. 11, 1920, 5.50 p.m. Injected intraspinally 2 cc. of fresh normal human serum. Mar. 12, 2.30 p.m. Injected intravenously 50 cc. of a 5 per cent suspension of fresh poliomyelitis nervous tissue. 2.50 p.m. Intraspinally injection of 2 cc. of fresh normal human serum. The intraspinal injection of 2 cc. of normal human serum was repeated daily for 3 days. Mar. 18. Excited; slight head tremor and left facial paralysis. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

*Experiment 2.*—*Macacus rhesus* B. Immune poliomyelitic serum. Mar. 11, 1920, 6 p.m. Injected intraspinally 2 cc. of serum from a monkey which had had experimental poliomyelitis 9 months before and had recovered with residual paralysis. Mar. 12, 3 p.m. Injected intravenously 50 cc. of virus suspension. 3.25 p.m. Intraspinally injection of 2 cc. of poliomyelitic immune monkey serum. The intraspinal injections of 2 cc. of immune monkey serum were repeated daily for 3 days. The monkey remained well.

*Experiment 3.*—*Macacus rhesus* C. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.05 p.m. Injected intraspinally 2 cc. of serum from Case 1, age 31 years, who was in the 5th week of well defined lethargic encephalitis with general disturbance of the functions of the central nervous system and involvement of third and seventh cranial nerves. Mar. 12, 3.30 p.m. Intravenous injection of 50 cc. of virus suspension. 3.55 p.m. Intraspinally injection

of 2 cc. of encephalitis serum from Case 1. The intraspinal injection of the encephalitic serum was repeated daily for 3 days. Mar. 17. Animal slow and weak. Mar. 18. Found dead at 9 a.m.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

*Experiment 4.*—*Macacus rhesus* D. Serum from convalescent case of lethargic encephalitis. Mar. 11, 1920, 6.10 p.m. Injected intraspinally 2 cc. of serum from Case 2, age 34 years, 3 months after definite attack of lethargic encephalitis in which there was general disturbance of the function of the central nervous system, involving the third and seventh cranial nerves and spinal motor roots. Mar. 12, 4 p.m. Intravenous injection of 50 cc. of virus suspension. 5 p.m. Injected intraspinally 2 cc. of convalescent encephalitis serum from Case 2. The intraspinal injection of 2 cc. of encephalitic serum was repeated daily for 3 days. Mar. 18. Excited and ataxic. Mar. 19. Prostrate. Mar. 20. Etherized when moribund.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

### *Series 2.*

The second series of tests carried out at a different time was controlled by an experiment in which normal human serum was used for the intraspinal injections as in Experiment 1. The procedure in this series was the same as in Series 1, except that normal horse serum was used for the preparatory intraspinal injection given the day before the intravenous injection of virus. The same strain of virus employed in Series 1 was again tested for infecting power and used in this series.

*Experiment 5.*—*Macacus rhesus* E. Normal human serum control. May 18, 1920, 4.10 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 12 m. Intravenous injection of 50 cc. of virus suspension. 12.25 p.m. Injected intraspinally 2 cc. of normal human serum. The intraspinal injection of normal human serum was repeated daily for 3 days. May 23. Monkey excited; both legs weak. May 24. Complete paralysis of both legs and right facial paralysis. May 25. Died.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

*Experiment 6.*—*Macacus rhesus* F. Serum from case of lethargic encephalitis. May 18, 1920, 4 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11.30 a.m. Intravenous injection of 50 cc. of virus suspension. 11.55 a.m. Intraspinal injection of 2 cc. of serum from Case 3, age 28 years, taken 4½ months after acute onset of lethargic encephalitis. The patient's illness began with dizziness, disturbance of vision, vomiting and fever, and he gradually became stuporous. Later paralyzes referable to the seventh and spinal nerves appeared. The intraspinal injection into the monkey of the convalescent serum was repeated

daily for 3 days. May 26. Monkey had paralysis of both arms and shoulder muscles. May 27. Died.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

*Experiment 7.*—*Macacus rhesus* G. Serum from case of lethargic encephalitis. May 18, 1920, 4.20 p.m. Injected intraspinally 2 cc. of normal horse serum. May 19, 11 a.m. Intravenous injection of 50 cc. of virus suspension. 11.25 a.m. Injected intraspinally 2 cc. of serum from Case 4, age 24 years, who had developed lethargic encephalitis 15 months before and was still under observation with residual ptosis and partial paralysis of left side of face and left leg. The intraspinal injection into the monkey of the convalescent encephalitis serum was repeated daily for 3 days. May 24. Monkey had double ptosis. May 25. Shoulder muscles paralyzed; arms and legs very weak; head tremor; animal almost prostrate. May 26. Died.

*Autopsy.*—Microscopic lesions of experimental poliomyelitis.

#### CONCLUSIONS.

Lethargic encephalitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the basal ganglia of the brain.

Poliomyelitis is an epidemic disease, the main manifestations of which relate to injury inflicted upon the central nervous system and in particular the gray matter of the spinal cord and medulla oblongata.

At the outset of the epidemic of lethargic encephalitis the two diseases tend to prevail at distinct and different seasons of the year, although recently cases of epidemic encephalitis have arisen in the midsummer months. The two maladies therefore are perhaps less distinguished by seasonal prevalence than has been supposed.

They are, however, distinguished by great differences in communicability to monkeys. Epidemic poliomyelitis is readily transmitted through inoculation of the affected central nervous tissues of man to monkeys, while it may still be regarded as doubtful whether lethargic encephalitis has been communicated to monkeys in this manner.

As the experiments reported in this paper show, the two diseases can be distinguished through the power of blood serum under certain circumstances to neutralize the virus of poliomyelitis. The blood serum of convalescent cases of poliomyelitis whether in man or monkey

possesses this neutralizing power, while the blood serum of recently convalescent cases of epidemic encephalitis is devoid of it.

On the basis of the distinguishing characters described, it is regarded as desirable at the present time to hold epidemic poliomyelitis and epidemic encephalitis as integrally distinct affections. The latter also may be infectious, yet the main lesions of poliomyelitis are present in the spinal cord, and of epidemic encephalitis in the mid-brain.

THERAPEUTIC ACTION OF N-PHENYLGLYCINEAMIDE-*p*-  
ARSONIC ACID (TRYPARSAMIDE) UPON EXPERI-  
MENTAL INFECTIONS OF TRYPANOSOMA  
RHODESIENSE.

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(Received for publication, October 13, 1920.)

In a series of papers published in November, 1919 (1), we reported results which had been obtained from the treatment of various forms of experimental trypanosomiasis with *N*-phenylglycineamide-*p*-arsonic acid, or tryparsamide. The results were such as to indicate that this drug might prove of value in the treatment of the form of human trypanosomiasis which is due to infection with *Tr. gambiense*. At the time this work was done, *Tr. rhodesiense* was not obtainable for experimental study, but as the therapy of the infections caused by this organism is becoming of increasing importance, it was deemed advisable to determine the effects which might be hoped for from the use of this drug in the treatment of Rhodesian sleeping sickness, and something of the method of treatment which might be employed in these cases.

Accordingly, a strain of *Tr. rhodesiense* was obtained and a series of experiments carried out upon various forms of the animal infection including that in mice, rats, and rabbits.

*Tr. rhodesiense* usually exhibits a higher degree of virulence for laboratory animals than *Tr. gambiense*. The organism used in these experiments, however, was distinctly less virulent for all the animals used, except the rabbit, than the strains of *gambiense* with which we had worked. After serial passage through mice and rats, it developed a fair degree of virulence for both species of animals and was highly virulent for rabbits, but possessed very slight virulence for guinea pigs. When inoculated into mice and rats according to the procedure described in a previous paper (2), the incubation period of the infection

was from 2 to 3 days in the case of mice and 3 to 5 days in rats. Infected mice died in from 10 to 12 days, while rats survived a few days longer. Rabbits were also readily infected; well marked symptomatic manifestations of disease developed in less than a week after intravenous inoculation, and the infection terminated fatally within 2 to 4 weeks.

Attempts to transmit the infection to guinea pigs were at first unsuccessful, but eventually a low grade infection with a prolonged incubation period and a low mortality was produced and serial passages were maintained through several generations of transfers.

#### EXPERIMENTAL.

The general plan of the therapeutic experiments carried out was the same as that employed in our previous work; that is, the trypanocidal action of the drug was studied by the use of the simpler blood stream infections of mice and rats, and the results thus obtained were applied to the treatment of the more complex disease as it appears in the rabbit. Mice and rats were inoculated intraperitoneally and treated 24 hours later by the intraperitoneal administration of a single dose of the drug. Repeated blood examinations were made to determine the effects upon the infecting organisms. Mice were kept under observation for 1 month after treatment and rats for 2 months, as a means of determining the probable results of treatment.

Rabbits were inoculated intravenously and treated by the same route when well marked symptoms of disease had developed; the period of observation was 3 months, although most of the animals in this series were held as long as 4 months.

#### *Therapeutic Effects.*

*Mice.*—Several series of mice were treated with doses of from 0.15 to 1.75 gm. per kilo of body weight. Doses below 0.25 gm. per kilo were found to exercise very little if any effect upon the general course of the infection and those of from 0.25 to 0.5 gm. merely served to delay its progress. No cures were obtained until the dose of drug given reached 0.75 to 1 gm. per kilo. As an example of the therapeutic effects obtained, the detailed results of one of the experiments are given in Table I.



TABLE I.

*Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Mice.\**

Dose per kilo.	No. of mice.	No. of relapses.	No. of probable cures.
<i>gm.</i>			
1.75	2	0	2
1.50	2	0	2
1.25	2	1	1
1.00	2	2	0
0.75	2	1	1

\* Controls survived 11 days.

*Rats.*—It was found that the rat infection of *Tr. rhodesiense* was also difficult to influence with this drug. The results obtained from experiments carried out under the same conditions as with mice may be illustrated by the experiment given in Table II.

TABLE II.

*Results from the Treatment of a 24 Hour Infection of Tr. rhodesiense in Rats.*

Dose per kilo.	No. of rats.	No. of relapses.	No. of probable cures
<i>gm.</i>			
0.75	3	0	3
0.60	3	2	1
0.50	3	1	2
0.35	3	3	0

While smaller unit doses of the drug were capable of curing the infection produced in rats than in mice, when considered from the standpoint of the ratio of the curative to the lethal dose, the figures obtained in the two instances were in very close agreement, with a slightly better ratio for the mouse. Thus it was found necessary to administer upwards of two-thirds of the maximum dose in order to effect a complete sterilization in the case of the mouse, and while cures might be obtained with only two-thirds of the maximum dose in rats, the full dose was necessary to assure such a result.

These figures were quite different from those which had been obtained with *Tr. gambiense* under similar conditions. Comparable results were obtained in mouse and rat infections of this organism

in doses as small as one-ninth and one-fifth respectively of the maximum dose for the two animal species. It appeared, therefore, that greater difficulty might be anticipated in the treatment of the more severe chronic infection of rabbits.

*Rabbits.*—In tests of the therapeutic effects against *rhodesiense* infection in rabbits, the animals used were inoculated intravenously with 1 cc. per kilo of body weight of a + to + + suspension of trypanosomes prepared from rat blood. This produced a very intense infection, symptoms appearing in individual animals within 3 to 5 days after inoculation, and the majority of the animals showed well marked manifestations of disease by the end of the 1st week. The controls of the series survived for only 2 weeks. Three types of experiments

TABLE III.

*Results from the Treatment of Tr. rhodesiense Infections in Rabbits by the Intravenous Administration of a Single Dose of Tryparsamide Given 1 Week after Inoculation.*

Dose per kilo.	No. of rabbits.	No. of relapses.	No. of probable cures.
<i>gm.</i>			
0.75	3	1*	2
0.60	3	2†	1

\* Relapse 19 days after treatment.

† Relapse 19 and 22 days after treatment.

were undertaken in this series: (1) the use of large single doses, (2) the use of repeated smaller doses, and (3) an intensive treatment of relapses.

*Single Doses.*—In view of the marked resistance exhibited by *Tr. rhodesiense* in mice and rats, the single dose treatment of rabbits was placed at the highest possible level consistent with safety. A small series of rabbits was treated, with the results recorded in Table III.

*Repeated Doses.*—The attempt was also made to determine whether any form of repeated dose therapy based upon the use of smaller doses than those described above might prove efficacious in this class of infection. Two rabbits were given doses of 0.4 gm. per kilo at intervals of 3 days. One of these received six doses, and after a rest

period of 1 week, the treatment was resumed with nine doses at intervals of 2 to 3 days. The animal thus received a total dose of 6.0 gm. of the drug per kilo of body weight within a period of 40 days. With the second animal the number of doses was reduced to six, or a total amount of 2.4 gm. of the drug per kilo was given within 15 days. Both these animals showed rapid improvement and remained in excellent condition throughout the course of treatment. Signs of the disease disappeared completely and there was no recurrence within the period of observation. They may, therefore, be regarded as probable cures.

The same procedure was followed with three other rabbits with a still smaller unit dose of the drug—0.3 gm. per kilo. One animal received six doses of the drug at intervals of 3 days, or a total of 1.8 gm. in 15 days; the second received five doses followed by a rest period of 1 week and then a second series of ten doses, or a total of 4.5 gm. was given in 43 days; the third rabbit was given fifteen consecutive doses at regular intervals, the 1 week interim between series being omitted.

The first of these animals did well under the treatment; symptoms of disease disappeared promptly and there was no recurrence. The initial effects were much the same in the second animal, but although the course of treatment was much more prolonged, it proved ineffectual. Within 4 days after administration of the drug had been stopped, signs of the disease reappeared, and the animal died from trypanosomiasis 15 days later. With the third rabbit, which was given the same amount of drug in consecutive doses, a cure was effected.

This small group of cases furnishes an excellent illustration of the variability of individual results which may be obtained in the treatment of infections of this type and possibly also the effects which may be produced by apparently minor variations in the course of treatment.

*Relapses.*—In view of the great difficulty experienced in influencing *rhodesiense* infections, little was to be expected from an attempt to treat these infections once they had relapsed. It was considered worth while determining, however, whether it was possible to obtain any effect from the use of the drug in this class of cases. Three experiments of this kind were carried out.

The first case treated was a rabbit which had relapsed from a single dose of 0.75 gm. per kilo. On the 3rd day after the symptoms of the disease reappeared, treatment was begun with 0.5 gm. per kilo, and nine doses of the drug were administered at intervals of 2 to 3 days, or 4.5 gm. per kilo in 21 days. This was considered to be as intensive treatment as could be undertaken and in this instance apparently effected a cure. The animal when inoculated weighed 1,625 gm.; when retreated after relapse, 1,600 gm.; when treatment was discontinued, 1,875 gm.; when discarded, 1,900 gm. This experiment furnishes an excellent example of the remarkable tolerance exhibited by animals towards the drug and the improvement in general condition which usually follows its administration.

A second animal which had relapsed from a single dose of 0.6 gm. was given five doses of 0.5 gm. and three doses of 0.6 gm. in 18 days, but the treatment was ineffectual and relapse occurred 1 week after it was discontinued.

In a third rabbit, the effect of the treatment was uncertain. This was likewise a case of relapse from a single dose of 0.6 gm. per kilo. The animal was in poor condition when relapse occurred and retreatment was instituted. Two doses of 0.5 gm. per kilo were given with 48 hours intervening. The signs of trypanosomiasis were but slightly improved, and the animal was weaker, so the dose of drug was reduced to 0.3 gm. and two doses of this size were administered. Death occurred 3 days later without complete disappearance of the manifestations of trypanosomiasis.

By comparing these results with those previously reported from the treatment of rabbits infected with *Tr. gambiense*, it is at once apparent that the *rhodesiense* infections were much more difficult to influence. Results comparable with those obtained with single doses of 0.6 to 0.75 gm. per kilo could be obtained in *gambiense* infections with approximately one-third of this amount of drug, and while cures were uniformly obtainable in *gambiense* infections with from 0.3 to 0.35 gm. per kilo, there appeared to be no single dose of the drug with which this could be accomplished with safety in cases of *rhodesiense* infection.

## CONCLUSIONS.

With the three classes of animal infections studied, the trypanocidal action of tryparsamide was found to be much less for *Tr. rhodesiense* than for *Tr. gambiense*, and a correspondingly greater difficulty was experienced in the treatment of the chronic tissue infections of *Tr. rhodesiense*.

This is, of course, entirely in accord with past experience in the treatment of human cases of trypanosomiasis due to the two organisms. It is still possible that something may be accomplished by the use of tryparsamide in cases of Rhodesian sleeping sickness on account of the tolerance exhibited to the drug and the possibility of employing an intensive system of treatment. Much less is to be expected, however, than in cases of infection due to *Tr. gambiense*.

## SUMMARY.

A series of experiments was carried out to determine the effects of tryparsamide upon the infections produced in various species of animals by *Tr. rhodesiense*.

The strain of trypanosome used was one which possessed a very low virulence for guinea pigs, was fairly virulent for mice and rats, and highly virulent for rabbits.

Therapeutic experiments carried out on 24 hour infections in mice and rats showed that cures could be obtained in this class of infections by the administration of a single large dose of the drug amounting to approximately two-thirds of the maximum tolerable dose, as contrasted with similar results in cases of *gambiense* infections from approximately one-ninth and one-fifth of this dose respectively.

With advanced infections in rabbits, there appeared to be no single dose of the drug capable of insuring a cure which could be administered with safety, although some cures were obtainable with doses approximating the maximum tolerable dose. Treatment of these infections could be carried to a successful conclusion, however, by an intensive system of treatment in which large doses of the drug were administered at short intervals of time, and even relapses yielded to this treatment in some instances. The employment of such a method of treatment was possible on account of the unusual tolerance exhibited by animals to this drug, a fact which was previously emphasized.

These facts indicate that the outlook for the drug in the therapy of Rhodesian sleeping sickness is much less hopeful than in *gambiense* infections, though it is felt that some benefits may be derived from its use.

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## STUDIES OF THE ACID-BASE EQUILIBRIUM IN DISEASE FROM THE POINT OF VIEW OF BLOOD GASES.\*

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(Received for publication, August 2, 1920.)

### INTRODUCTION.

The problem of acidosis resolves itself into two fundamental questions. First, in any given case is there an acidosis in the sense that the available alkali of the blood is diminished through an increase in non-volatile acid; and, second, is there an acidosis in the sense of an actual change in blood reaction in the acid direction, of an increase, in other words, of the concentration of ionized hydrogen? In disease these conditions may occur alone or combined. To recognize each and to distinguish between them is of some clinical importance. Data concerning the former are abundant in the literature; concerning the latter, somewhat less so; and data showing the two simultaneously are few.

The question of available alkali of the blood in disease has been approached by a large number of investigators and by divergent methods. Of the more recent work the following general types may be cited by way of illustration: (a) determination of titratable alkalinity of the blood by the method of Sellards (1); (b) determination of the carbon dioxide capacity of the serum by the methods of Van Slyke (2); (c) determination of the oxygen-combining power of the blood at a given tension of oxygen as done by Barcroft (3).

The question of the actual reaction of the blood in health and disease has also been approached in several ways. The reaction can be measured directly either electrometrically by the so called gas chain method of Hasselbalch (4) or by the use of indicators as employed by Sørensen (5) and more recently by Bayliss (6). It can also be arrived at indirectly from the behavior of the oxygen dissociation curve of the blood, the determination of the degree of meionexy of Barcroft (3).

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\* This paper is the first of a series of studies of the physiology and pathology of the blood from the Harvard Medical School and allied hospitals.

Finally it may be calculated from the carbon dioxide dissociation curve provided that the arterial carbon dioxide tension or concentration is known. Hasselbalch (7) has published a formula for such calculation which is dependent upon the principle shown by L. J. Henderson (8) that the reaction of the blood is dependent chiefly upon the ratio of free carbonic acid to bicarbonate, which can be expressed by the formula

$$[H]^+ = K \frac{[H_2CO_3]}{[BHCO_3]}$$

in which  $[H]^+$  is the concentration of ionized hydrogen,  $[H_2CO_3]$  the concentration of free carbonic acid and  $[BHCO_3]$  the concentration of bicarbonates, and  $K$  a constant. The fraction  $\frac{[H_2CO_3]}{[BHCO_3]}$  ordinarily has a value of approximately  $\frac{1}{20}$ . Changes in this ratio, it can easily be seen, will denote changes in the  $[H]^+$ , that is in blood reaction, while changes in the actual magnitude of the denominator will denote changes in available alkali of the blood and changes in the numerator changes in carbon dioxide tension. Knowledge, then, of the two terms of the ratio in any given blood will tell us the two most fundamental facts of acidosis.

For clinical purposes the simplest way of arriving at the value of the ratio and of determining the actual magnitude of the two terms of the fraction is to secure the data necessary to plot the so called carbon dioxide diagram of the blood of Haggard and Y. Henderson (9). This diagram, which incidentally is essentially a graphic representation of L. J. Henderson's formula, consists in the carbon dioxide dissociation curve of the blood determined after the manner of Christiansen, Douglas, and Haldane (10) with a mark upon it which denotes its intersection with the coordinate representing the tension or concentration of carbon dioxide in the arterial blood, this point being called the A-point.

After a conversation with Professor Y. Henderson in January, 1920, we were greatly impressed with the lucidity of such diagrams in the interpretation of problems of acid-base equilibrium, and as a result we undertook the present research which consists in the construction of such diagrams for the blood of patients, for the purpose of determining the presence or absence of non-volatile acid in their blood, and of actual changes in their blood reaction.

The information to be gathered from the dissociation curve thus plotted with its A-point is as follows: In the first place the abscissæ in the diagram indicate the tension of carbon dioxide in the blood and therefore the concentration of dissolved carbonic acid, for it is well known that the concentration of carbonic acid in physical solution is directly proportional to its partial pressure, or tension. The abscissa



therefore is the numerator of the fraction in L. J. Henderson's formula  $[H_2CO_3]$ . Second, the ordinates are indices of the denominator of the fraction, or the concentration of bicarbonates in the blood  $[BHCO_3]$ . For although what is actually determined is the total carbonic acid of the blood, not only in chemical combination as bicarbonate, but in physical solution as well, yet, since the latter is directly proportional to the tension, it can be disregarded. Indeed Haggard and Henderson in their diagram by drawing a diagonal base-line show directly the amount of carbon dioxide in physical solution and chemically combined. We have not used this method, as in the majority of curves in the literature the total carbonic acid at any given tension has usually been plotted above a horizontal base-line.

If now the abscissa represents dissolved carbonic acid concentration and the ordinate bicarbonate concentration, and if hydrogen ion concentration is dependent upon the ratio of these two to each other multiplied by a constant, then for any given point in the diagram a series of radii drawn through the zero point will indicate the magnitude of the hydrogen ion concentration. Also in any given arterial blood the position of the A-point will show the reaction. As long as the value of the ratio  $[H_2CO_3] : [BHCO_3]$  remains constant, though its actual terms may vary in magnitude, the A-point will always fall on the same straight line drawn through the zero point. We have therefore drawn a diagonal, the line OC in Text-fig. 1, which runs approximately through the A-points of a number of normal bloods. Pathological bloods, if the A-points fall to the left of this line, will have a more alkaline, if to the right a less alkaline reaction than normal.

By the carbon dioxide diagram then we are informed, first, of the presence or the absence of acidosis in the sense of a changed blood reaction, and, second, of the presence or absence of non-volatile acid in the blood. This latter is shown by the level of the dissociation curve. The effect of adding acid to the blood is to diminish its available alkali. This means that the dissociation curve will be moved downward, for the ordinate represents the concentration of blood bicarbonate. The two main facts of acidosis then are revealed by the diagram, through the position of the A-point and the level of the curve.

*Methods.*

There is nothing new in the methods we have made use of. Blood was drawn from an arm vein and placed in a tube containing some dry oxalate crystals. About 5 cc. were placed in each of three tonometers which had previously been filled with mixtures of air and carbon dioxide of known tension. The type of tonometer<sup>1</sup> used was that shown by Barcroft (3). The tonometers were then placed in a brass cylinder filled with water at body temperature and slowly rotated in a horizontal position for 10 minutes by a motor.<sup>2</sup> After equilibration the carbon dioxide content of the blood from each tonometer was determined by means of Henderson and Smith's blood gas apparatus (11).

Three points in the dissociation curve were thus obtained and plotted as in Text-fig. 1. The ordinates represent the concentration of carbon dioxide of the blood in terms of volumes per cent; that is, the content as found by blood gas analysis. The abscissæ represent the tension of carbon dioxide in millimeters of mercury; that is, the partial pressure of carbon dioxide in the equilibrating atmosphere. These points having been obtained for any given blood, the most probable curve was then drawn through them.

The next step in the construction of the carbon dioxide diagram is the fixing of the arterial or A-point. This can be done, of course, by finding either the concentration of carbon dioxide in the arterial blood, its appropriate abscissa, and the point where this intersects the curve, or by determining the tension of carbon dioxide in the arterial blood and finding the point where its ordinate intersects the curve. If both methods were used they would serve as a check upon each other.

The concentration of carbon dioxide in arterial blood we obtained by drawing samples under oil from the radial or brachial artery after the method of Stadie (12), and then determining their carbon dioxide content with the Henderson apparatus. Direct determination of the tension of gases in the circulating blood of animals has been done experimentally by Krogh and Krogh (13), but could hardly be done

<sup>1</sup> Barcroft (3), Fig. 145.

<sup>2</sup> This rotating bath was a copy of one which was in use in Barcroft's laboratory at Cambridge in 1913.

satisfactorily in man. In the absence of lesions of the lungs or of disturbances of the circulation through the pulmonary circuit, however, it is safe to assume that the arterial blood, as far as carbon dioxide is concerned, is essentially in tension equilibrium with the alveolar air, as obtained by Haldane and Priestley's method (14).

In plotting the A-point of our several curves we sometimes have made use of the carbon dioxide tension of the alveolar air, and sometimes of the carbon dioxide content of the arterial blood. The method used in each instance is noted in Table I.

### *Cases Studied.*

We determined, in all, fifteen dissociation curves. All the points determined are shown in Table I, while the actual curves with their A-points are shown in Text-fig. 1. Two of these curves were of the blood of normal individuals, three of a diabetic with acidosis, two of a nephritic with acidosis, three of pneumonia patients, two of anemia (one primary and one secondary), one of cerebral hemorrhage, one of myelogenous leucemia, and one of idiopathic tetany.

*Normal Individuals.*—As we stated earlier in interpreting the carbon dioxide diagram one phase of the question is answered by the level of the dissociation curve. Since this is true, it becomes necessary to discover the level of the normal curve. We have searched the literature for such curves and found several. In Text-fig. 1 a shaded zone is shown. This zone contains curves of the blood taken from the work of Christiansen, Douglas, and Haldane (10), of Hasselbalch (7), of Parsons (15), of Joffe and Poulton (16), of Liljestrand and Lindhard (17), and also curves of two cases described in the present paper, Cases A and B (Curves 1 and 2). A similar zone has been published by Straub and Meier (18), which is said to include 320 points of the bloods of 64 normal persons. The lower border of their zone falls within our zone, but their upper border is very much higher and so at variance with the work of other investigators that we are inclined to believe it incorrect. All the A-points of the blood of Cases A and B and of those determined by Christiansen, Douglas, and Haldane, and Liljestrand and Lindhard fall either on the OC line in Text-fig. 1, or at a distance on either side of it so slight that it would

TABLE I.  
*Dissociation Curve; Points Actually Determined.*

Curve No.	Case.	Condition.	CO <sub>2</sub> tension.	CO <sub>2</sub> content.	A-point.
			<i>mm.</i>	<i>vol. per cent</i>	<i>mm.</i>
1	A	Normal.	16.8	38.0	37.5
			41.0	51.0	
			72.0	61.0	
2	B	"	17.6	37.0	40.6
			36.0	49.0	
			65.0	59.0	
3	C	Diabetic acidosis.	19.5	19.0	14.6
			42.5	27.0	
			74.0	39.5	
4	C	Same. 1 day later.	18.1	21.0	20.5
			38.2	30.0	
			78.2	39.0	
5	C	" 3 days after Curve 4 was determined.	18.5	Lost.	26.8
			42.5	41.0	
			67.0	48.0	
6	D	Renal acidosis.	17.9	13.0	<i>per cent</i> 12
			32.5	22.0	
			67.0	36.0	
7	D	Same. Day after alkali administration.	18.5	45.0	60
			35.4	62.0	
			69.5	72.0	
8	E	Influenza pneumonia.	19.7	38.0	57
			31.5	44.0	
			68.0	61.0	
9	F	Lobar pneumonia.	18.5	34.0	52
			32.5	46.0	
			67.5	58.0	
10	G	Bronchopneumonia.	21.8	50.0	68
			39.4	58.0	
			69.5	70.0	

TABLE I—*Concluded.*

Curve No.	Case.	Condition.	CO <sub>2</sub> tension.	CO <sub>2</sub> content.	A-point.
			<i>mm.</i>	<i>vol. per cent</i>	<i>per cent</i>
11	H	Cerebral hemorrhage.	22.0	46.0	56
			34.5	54.0	
			69.2	65.0	
12	I	Myelogenous leucemia; secondary anemia.	18.9	47.0	40.6
			42.6	62.0	
			80.5	72.0	
13	J	Secondary anemia.	17.7	42.0	Lost.
			36.6	50.0	
			70.5	63.0	
14	K	Idiopathic tetany.	18.8	43.0	58
			38.2	53.0	
			70.0	66.0	
15	L	Primary anemia.	19.5	55.0	70
			35.8	69.0	
			73.8	74.0	

make a difference of not more than 0.02 in the pH. The data for placing the A-point of the other curves taken from the literature were not found in the several papers.

*Diabetic Acidosis.*—Three curves (Nos. 3, 4, and 5) were obtained from a case of diabetic acidosis (Case C).

The first of these curves (No. 3) was obtained on January 23, 1920. The patient had then just entered the hospital with a moderately severe diabetes mellitus, and with a considerable acetonemia. He was somewhat drowsy but not in coma. There was no noticeable hyperpnea and no subjective dyspnea. The low position of the curve on this day shows a very low combining power of the blood with carbon dioxide and hence indicates the presence of a large quantity of non-volatile acid in the blood. Nevertheless, the A-point of this curve is practically on the OC line, which means presumably that his blood reaction was normal. He had, in other words, what has been called by Y. Henderson and others a compensated acidosis. He had acidosis

in the sense of having abnormal acid present in his blood, but he had by increasing his pulmonary ventilation and thereby reducing the tension of carbon dioxide in his alveolar air so reduced the dissolved carbon dioxide of his blood that the normal value of the  $[H_2CO_3] : [BHCO_3]$  ratio was preserved. Through his pulmonary response he had kept his blood reaction normal.

After this he fasted, but was given water and salt freely, and on January 24 his dissociation curve had risen to the position of Curve 4. The A-point of this curve is slightly to the acid side of the 0C line and may indicate that his compensation was not quite so good as it had been the day before. It must be said, however, that as we approach the lower left-hand corner of the diagram the position of the A-point becomes less and less accurate. The experimental error in determining the carbon dioxide content by the method used is probably at least 1 per cent, and with low curves an error of 1 per cent will make much more change in the pH than it would with curves in the normal position. We should hesitate to say then that the A-point of Curve 4 really proved the presence of a decompensated acidosis. The fact that Curve 4 is higher in position than Curve 3 shows that the patient had somewhat improved his supply of available blood alkali.

On January 27, Curve 5 was obtained. This shows a very striking increase in available alkali and an A-point on the 0C line, indicating perfect compensation.

Unfortunately no further observations were obtained with this patient, for he left the hospital against advice.

*Nephritic Acidosis.*—We also had an opportunity to obtain two curves in the case of a man, 55 years old (Case D), who was brought to the hospital with an intense grade of hyperpnea. It proved to be impossible to measure his pulmonary ventilation, but from the general character of his breathing as compared with a similar case of hyperpnea reported by Means and Rogers (19) it seemed probable that he was breathing 40 to 50 liters per minute.

His underlying malady was arteriosclerotic nephritis but he had in addition an obstructing prostate and a double pyelonephritis. He was in a profound state of uremia and retention acidosis. The dissociation curve (Curve 6) obtained the day he came in, February 19, 1920,

had the lowest position of any in the series, indicating that the available alkali was very greatly reduced. Moreover, the position of the A-point of the curve definitely to the right of the OC line shows that, in spite of the extreme hyperpnea, the respiratory response was insufficient to preserve the normal  $[\text{H}_2\text{CO}_3] : [\text{BHCO}_3]$  ratio and he had an actual change in the reaction of his blood in the acid direction, a decompensated acidosis in other words. The actual shift in the pH, as explained earlier, in this part of the diagram can only be roughly estimated, but it could not have been less than 0.2. His actual oxygen saturation on this day was 89.6 per cent and his hemoglobin 69.3 per cent (calculated from the oxygen capacity).

After the blood had been drawn for the first curve he was promptly started on large amounts of sodium bicarbonate intravenously and by rectum. A free flow of urine was also obtained by catheterization.

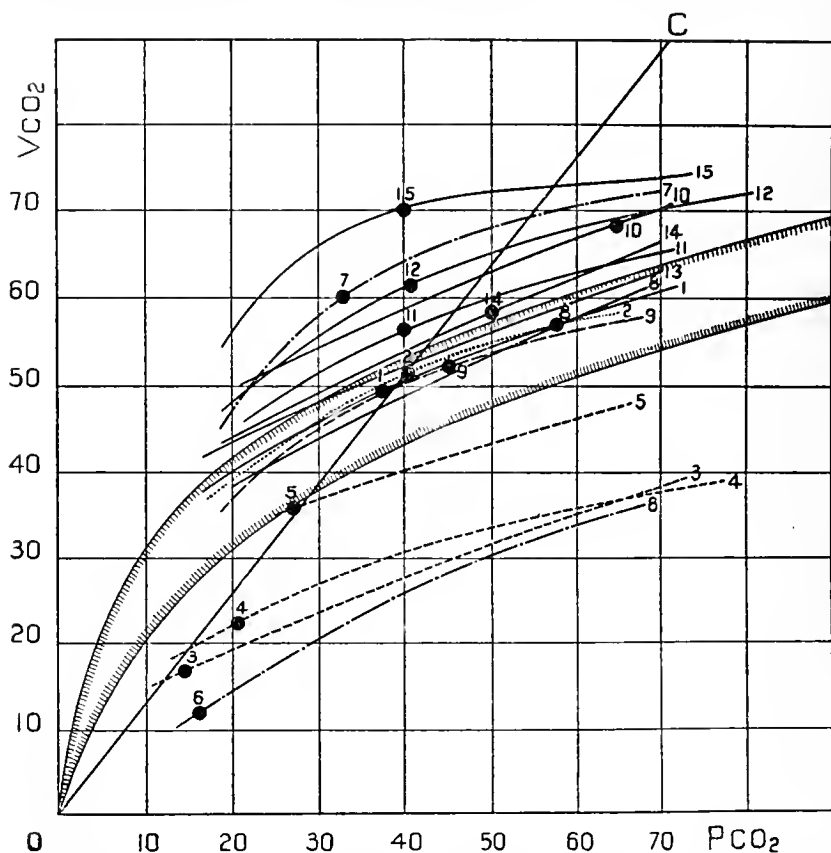
The next day, after he had had approximately 175 gm. of sodium bicarbonate, there was a most extraordinary change. The hyperpnea had entirely gone and he was no longer drowsy. Curve 7 was obtained. The change in the curve is as remarkable as that in the clinical condition. From being very low it had moved to a level higher than normal. On that day, then, the available alkali was greater than in normal individuals. Furthermore, the A-point had moved to the left side of the OC line to such a distance that he probably had, on that day, an actual shift in blood reaction in the alkaline direction. A large amount of fluid had been administered to him and this was reflected in the hemoglobin, which had dropped to 51.1 per cent. Moreover, he no longer had arterial anoxemia, his arterial blood being 93.5 per cent saturated.

The improvement, of course, was transient, as he had practically no renal tissue. He became uremic and acidotic again, and died on February 23.

*Pneumonia.*—A curve was obtained from each of three patients with pneumonia.

The first of these (Curve 8) was that of the blood of a man, 39 years old, with influenza pneumonia (Case E). The curve was obtained on February 18, 1920. The influenza began on February 12 and the pneumonia on February 16. It was of the fulminating variety of influenza pneumonia. On the day the curve was obtained there were

scattered areas of consolidation throughout both lungs. There were frothy bloody expectoration and a marked diffuse bronchitis. Respirations were rapid and shallow. He had a dull, leaden cyanosis of the mucous membranes and finger tips. The striking point about the diagram of this case is that while the level of the curve is quite within the normal zone, the A-point is far to the right of the OC line, indicating



TEXT-FIG. 1. Carbon dioxide diagram in health and disease. The ordinates represent carbon dioxide content of the blood in volumes per cent ( $V\text{ CO}_2$ ), the abscissæ, tension of carbon dioxide in millimeters of mercury ( $\text{PCO}_2$ ). The shaded zone indicates the area within which we believe the curves of normal blood fall. The line OC represents the hydrogen ion concentration of normal blood. The arterial or A-points are shown by black dots. The points in the several curves actually determined, and also the condition of the patients, are shown in Table I.



a definite shift in the blood reaction in the acid direction, equal roughly to a decrease of about 0.10 in the pH. On the same day the oxygen saturation of the arterial blood of this case was 59.3 per cent and the hemoglobin 103.8 per cent. The arterial blood as drawn into a syringe was quite as dark as normal venous blood. He died on February 19. No autopsy was obtained.

The next diagram of pneumonia blood (Curve 9) is that of a man, 59 years old, with true pneumococcus (Type IIa) lobar pneumonia (Case F). The curve was obtained on February 11, 1920, the pneumonia having begun about 2 weeks before. He had on this day extensive double lobar pneumonia, the course of which had been progressively worse. His breathing was labored and grunting and he had marked cyanosis. About 8 hours after the blood for the diagram was taken he died. No autopsy was obtained. The arterial oxygen saturation on February 11 was 74.5 per cent and the hemoglobin 118 per cent. This curve, like the preceding one, is within the normal zone, the A-point also being to the right of the OC line, but less strikingly so than with the blood of Case E.

The last pneumonia blood studied (Curve 10) was that of a man, 43 years old, with postoperative bronchopneumonia. He also had pulmonary emphysema and chronic bronchitis (Case G). He was operated on for gall stones on February 21, 1920, and on February 22 the pneumonia began. The curve was obtained on February 25. On that day there were areas of consolidation scattered through both lungs, many râles, deep cyanosis, and rapid grunting breathing. It is interesting to note that the level of the dissociation curve is above the normal zone, yet the A-point like that of Curve 8 is well to the right of the OC line. The arterial oxygen saturation on February 25 was 86.3 per cent and the hemoglobin 122 per cent. He made an uneventful recovery.

*Blood Disease.*—The curves for the blood of three patients with blood disease were obtained. One of these (Curve 12) was that of a man, 33 years old, in an aleucemic phase of myelogenous leucemia; he likewise had a marked secondary anemia. The red cell count was 1,280,000, the white cell count 5,600, and the hemoglobin 45 per cent. The interesting thing about his curve is that it lies throughout at a higher level than the upper border of the normal zone and that the A-point lies to the left of the OC line.

Curve 13 of a man, 44 years old, with secondary anemia of moderate grade (Case J) whose red cell count was 3,248,000, white cell count 6,600, and hemoglobin 73 per cent also lay at a higher level than normal. Data for plotting the A-point of this blood were unfortunately not obtained.

The diagram of the blood of a man, 56 years old (Case L), with pernicious anemia (Curve 15) showed a still higher position of the dissociation curve and an A-point still further to the left than that of Curve 12. The red cell count was 1,849,000, the white cell count 4,000, and the hemoglobin 25 per cent.

*Miscellaneous Curves.*—One diagram (Curve 11) was obtained from a man, 58 years old, with a cerebral hemorrhage (Case H). This showed a slightly higher level of the curve than did the normal curves, and an A-point slightly to the left of the OC line.

The blood of a woman, 28 years old, with idiopathic tetany (Case K) showed a curve just above the upper border of the normal zone (Curve 14), and an A-point slightly to the right of the OC line.

#### DISCUSSION.

The interpretation of these results presents considerable difficulty. As we pointed out earlier, even the range of variation of the level of the carbon dioxide dissociation curve of normal blood is not known. The zone we have shown in Text-fig. 1, while it includes the curves determined for normal blood by a number of different observers, may perhaps not represent the true range. It may be either too wide or too narrow, or either or both of its borders may be too high or too low. It will require a large series of normal curves to determine correctly the limits of normal variation. Nevertheless, it seems to us that the striking variations from the few normal curves available, shown by some of the pathological bloods here studied, are not without clinical interest.

Before discussing these variations, we shall consider some of the possible sources of error. In the first place, what are the relative degrees of accuracy of the several procedures involved in the construction of the carbon dioxide diagrams? The shape and level of the dissociation curves we believe are substantially correct. The concentration

of carbon dioxide in the blood by the method used should be accurate within 1 volume per cent; that of carbon dioxide tension should certainly be accurate within less than 1 mm.

The position of the OC line which we have drawn to represent the normal ratio of free carbonic acid to bicarbonate according to the calculations of Straub and Meier from Hasselbalch's formula would indicate a pH of approximately 7.35. The actual pH of arterial blood as found by the gas chain method is given by Michaelis (20) as 7.45. As already stated, the A-point of the curves of the blood determinations in cases described by Haldane, Liljestrand, and Lindhard, and in Cases A and B of the present paper falls either on the line, as we have drawn it, or at an insignificant distance from it.

About the accuracy of the position of the A-points of the several curves we must speak with less assurance. As has already been said, two ways of plotting this point were used. Both these methods are open to criticism. The plotting of the A-point from the carbon dioxide tension of the alveolar air would be satisfactory provided, first, that accurate samples of arterial pulmonary air could always be secured, and, second, that the arterial blood is always in tension equilibrium with the alveolar air. In normal individuals both these provisions hold true. It is undoubtedly possible to obtain accurate alveolar carbon dioxide tension by Haldane's method in well trained normal subjects. So also it is entirely probable that their arterial blood is in tension equilibrium with their alveolar air, as far as carbon dioxide is concerned. In the sick patient, however, not only may it be impossible to obtain reliable Haldane samples of arterial pulmonary air, but furthermore, if there is a gross pathological condition within the thorax, it is quite possible that alveolar air and arterial blood will not be in tension equilibrium.

It was for this reason that in patients with pulmonary disease, or with those from whom for any reason we could not get good alveolar air samples, we resorted to the method of plotting the A-point from the carbon dioxide content of the arterial blood. This method might at first be thought to be free from the errors of the alveolar air method, but yet, as Peters (21) has recently pointed out to us, it is not entirely so. Provided the patient made no alteration in his pulmonary ventilation while the arterial sample was being drawn, there would be no

error, but if he either increased or decreased his ventilation, then we should have an abnormal carbon dioxide content, abnormally low in the former case, abnormally high in the latter, which would give us a false position of the A-point, too far to the left in the former case, too far to the right in the latter.

Another type of possible error is due to the nature of the diagram itself. In places where the dissociation curve is more nearly horizontal than vertical an A-point plotted from the carbon dioxide tension will obviously be more accurate than one plotted from the carbon dioxide content; and conversely, when the curve is more nearly vertical than horizontal, the carbon dioxide content will give the more accurate point. To illustrate this principle take for example the A-point of Curve 15. Here an increase of 1 volume per cent in carbon dioxide content would move the A-point some three times nearer the OC line than would a rise of 1 mm. in the tension, and a rise of 2.5 volumes per cent in the content would place it on the OC line, while a rise of 2.5 mm. in tension would still leave it far to the left.

One last source of error must be mentioned, and that is the effect of oxygenation upon the level of the carbon dioxide dissociation curve. Christiansen, Douglas, and Haldane (10) in their original paper showed that the curve for oxygenated blood has a definitely lower level than that of reduced blood. The curves that we have included in our normal zone were all those of oxygenated blood. Since arterial blood is normally nearly saturated no error is introduced in the plotting of the A-point by the effect of oxygenation on the carbon dioxide capacity except with such patients as have an arterial anoxemia. It will be noted that all three pneumonia patients studied had arterial anoxemia. It might be argued that in these cases it was not legitimate for us to place an A-point from the carbon dioxide content of their anoxic arterial blood upon a dissociation curve of fully oxygenated blood. This objection would hold except for the interesting fact just discovered by Y. Henderson that the dissociation curve of oxalated blood (that has not been exposed to air) usually shows no shift with oxygenation (22). The shift described by Christiansen, Douglas, and Haldane occurred in defibrinated blood. This finding of Y. Henderson has, we understand, been confirmed by L. J. Henderson (23). Its explanation is yet to be discovered, but

it enables us in the present research to eliminate the effect of oxygenation and reduction, and therefore to plot our A-points upon a curve of fully oxygenated blood, even though they are plotted from carbon dioxide contents of anoxemic bloods.

We shall now discuss the interpretation of our diagrams. We might repeat that our main object is twofold: first, to discover acidosis in the sense of diminished alkali reserve as shown by the level of the curve, and, second, to discover acidosis in the sense of altered blood reaction as shown by the position of the A-point; to distinguish, in other words, between acidosis compensated and acidosis decompensated, and of course to discover the opposite conditions of compensated and decompensated alkalosis if such exist.

The curves obtained with the diabetic patient (Curves 3, 4, and 5) show nothing particularly new. They illustrate clearly a condition of marked but compensated acidosis; that is, great reduction in available alkali but perfect pulmonary response with maintenance of normal blood reaction, and recovery therefrom.

The curves obtained with the nephritic case are even more striking. When the first curve (Curve 6) was obtained the patient undoubtedly was suffering from a decompensated acidosis. The change in the position of the curve over night, chiefly due no doubt to the alkali he had received, is very remarkable. From a condition of decompensated acidosis he had passed really in this brief interval to one of decompensated alkalosis, to use the same terminology.

The curves of the three pneumonia patients are perhaps more illuminating. Two of them (Curves 8 and 9) are within the normal zone, showing that both these patients had a normal reserve of alkali and therefore presumably no abnormal acid in the blood. With both of them the A-point is displaced in the acid direction. The curve of the third pneumonia patient (Case G) is at a higher level than normal, but like the other two its A-point is displaced to the right. These A-points are plotted from the carbon dioxide content of the arterial blood. As to their accuracy, we have already pointed out the effect of over or under ventilation. We have no proof that the ventilation of these patients was unaltered while we drew the arterial blood samples. However, they were all very ill, and took little or no interest in the procedure. Cocainization prevented pain, and therefore there

seems good reason to believe that no alteration in breathing occurred. There was no holding of the breath and no decrease in rate of breathing, none at least that could be told by observation. If any change occurred then it was more likely an increase than a decrease. But an increase would move the A-point to the left, which is the opposite to what was actually found. All things considered then, we feel that it is reasonably certain that the hydrogen ion concentration of the pneumonia blood was really altered in the acid direction.

In pneumonia then we may have in critically ill patients a condition in which although the buffer of the blood is quite normal, the reaction is more nearly acid than normally. There is an acidosis in the latter sense, but none in the former. The only explanation of this rather paradoxical state of affairs that occurs to us is that the condition actually is one of carbonic acidosis. A person with no non-volatile acid present in the blood, but with a pulmonary ventilation insufficient to preserve the normal  $[\text{H}_2\text{CO}_3] : [\text{BHCO}_3]$  ratio would have just such a diagram as these pneumonia patients. We already know because of their arterial anoxemia that they are not getting oxygen properly into their blood. It is certainly conceivable that in a similar way they are not getting carbon dioxide out. Their effective lung ventilation may be much greater than normal and still be insufficient, and it is not difficult to see how, because of mechanical limitations to the respiratory movements from pleurisy or through consolidation of the lungs, or because of decreased permeability of the alveolar membrane, the output of carbon dioxide might be hindered.

Henderson and Haggard (24) have shown that the reaction to increased carbon dioxide is an increase in the carbon dioxide capacity. This they observed in experimental morphine poisoning. It is the converse of the reaction to non-volatile acidosis. In the one case the available alkali being reduced, the tension of carbon dioxide is reduced in compensation, while in the other case the tension being increased the alkali is increased in compensation. It occurred to us that this might be what was happening in Case G (Curve 10). This curve is at a higher level than normal, yet the A-point is well to the right of the 0C line. The reaction of the blood then is more nearly acid than it should be, yet the available alkali is greater than normal also. This may be an attempt at compensation, and it can be seen that if the car-

bon dioxide capacity of this blood increased only 3 volumes per cent more, the A-point would fall on the OC line. It is probably only a coincidence, but it is interesting to note that this attempt at the compensation of a carbonic acidosis in this particular case proved a good prognostic sign, for this patient recovered shortly afterwards, while the other two died.

Of the curves of the three cases of blood disease (Curves 12, 13, and 15), two showed a higher level than normal, that of Case L with primary anemia being in the highest position of all the curves obtained. The curve of Case I with myelogenous leucemia in an aleucemic stage and with a marked secondary anemia, was also at a higher level than normal. The A-points of these two curves are also to the left of the OC line, denoting an increased alkalinity of the blood. The curve of the blood of the other anemic patient (Case J) is within the normal zone; the A-point of this curve was not obtained.

What may be the significance of the high level of these curves in anemia we do not know. Peters (21) tells us that he has found much the same thing in some of the anemic blood he has studied. The dissociation curve of the blood plasma lies at a higher level than does that of whole blood as shown by Hasselbalch (7) and by Joffe and Poulton (16), and it may be that the levels of anemic blood are higher in part because of the greater proportion of plasma that they contain. For the position of the A-points of these curves we also can offer no explanation. Both Curves 12 and 15 are more nearly horizontal than vertical near the OC line. Hence an A-point plotted from tension would be more accurate than one plotted from content. That of No. 12 was plotted from tension and is, we believe, reasonably accurate. That of No. 15 was plotted from content.

The two remaining curves are those of Case K with idiopathic tetany (Curve 14) and of Case H with cerebral hemorrhage (Curve 11). The former was constructed to see whether any evidence of a condition of alkalosis could be found. None was found, for the level of the curve is just above the upper limit of the normal zone and the A-point on the right, not on the left, of the OC line. The curve of Case H is, on the other hand, a little high and the A-point slightly to the left. The single observation in tetany does not prove very much for, as has been shown by Wilson, Stearns, and Thurlow (25), there are marked

and rapid variations in the acid-base equilibrium in tetany, and it is possible that our patient may have been changing from a stage of alkalosis to one of acidosis at the time the blood was drawn.

Considering the A-points of the entire series, we find that they are so distributed as to indicate a range of variation in the pH of roughly from 7.25 to 7.55. Sonne and Jarlöv (26) calculating in a similar way found a range of variation from 7.15 to 7.40, the difference in the pH of our extremes thus being 0.30 and of theirs 0.25. Their highest hydrogen ion concentration was in a case of severe diabetes mellitus with acetonuria, and their lowest was in a case of chronic nephritis with hypertension. With the gas chain method Michaelis (20) has found variations from 7.74 to 7.12, the first being in a case of syphilitic hemiplegia, the second in a case of diabetic coma just before death. It seems entirely likely then that our A-points are all within a range of variation compatible with life, and that they are not necessarily artifacts.

As to therapeutic indications brought out by the present study, there are one or two that are worth mentioning, in the first place in regard to acidosis such as that presented by the diabetic (Case C) and the nephritic (Case D). While the diminution of alkali was of somewhat the same order of magnitude, the acidosis of Case C was apparently compensated, while that of Case D was decompensated. It is also of interest that while Case C had no appreciable amount of respiratory distress Case D had a maximum amount. This we believe may in part have been due to the fact that the latter was a case of decompensated, while the former was one of compensated acidosis. This brings up the problem of what are the indications for alkali therapy. The curve of Case C went back towards normal with no alkali administration in a perfectly satisfactory way, and indeed it is generally conceded now that alkali is usually unnecessary in the treatment of diabetic acidosis.

In compensated acidosis the return to a normal level will be accomplished by the elimination of neutralized acid by the kidney. The lungs have already done their part in preserving blood reaction. Under the circumstances it is questionable whether the giving of alkali will accomplish any great good, and indeed it is possible that if too much is given it may do harm by producing alkalosis. The indica-



tion then in compensated acidosis would seem to be rather to promote elimination by the kidneys by giving fluids freely, and, if, as in diabetes, this is coupled with fasting which would lessen the further production of non-volatile acid, it would seem to be sufficient therapy.

In decompensated acidosis, on the other hand, alkali administration is probably indicated, because here there is an insufficient pulmonary response. The supply of additional bicarbonate may well restore the  $[H_2CO_3] : [BHCO_3]$  ratio to a normal value; indeed with Case D it did more than that and actually pushed his reaction to the other extreme. That it is desirable to restore blood reaction to normal cannot be doubted. All the high hydrogen ion concentrations have been found in critically ill patients, who, in fact, were usually in a moribund state. On the other hand, it is undoubtedly quite as important not to overdo the alkali therapy, as was done with Case D. A warning that excessive alkali therapy may be harmful has been given by Allen, Stillman, and Fitz (27). They believe, particularly in persons with damaged kidneys, that alkali should be used with great caution. That tetany can be produced after alkali administration is in itself evidence that alkalosis as well as acidosis may be harmful *per se* and equally to be avoided. The case reported by Harrop (28) of a woman with mercuric chloride poisoning who developed tetany and showed a plasma bicarbonate capacity of 80 volumes per cent after the intravenous injection of 60 gm. of sodium bicarbonate is particularly interesting in this regard. Equally pertinent is the recent work of Grant and Goldman (29), who succeeded in producing tetany and other unpleasant symptoms in themselves by prolonged voluntary forced breathing. They were able at the same time to demonstrate a change in the pH of the blood in the alkaline direction, and had the interesting situation of a low position of the dissociation curve as shown by the alveolar carbon dioxide tension and the carbon dioxide capacity of the plasma, and at the same time a blood more alkaline in reaction than normally. It really is the diametrically opposite condition to that shown by our pneumonia patient, Case G (Curve 10).

As to the indications for treatment in pneumonia, we can only say that they would seem to be to facilitate lung ventilation. According to our present knowledge, however, such indications can only be indirectly met. Measures which facilitate the mechanics of respiration,

the relief of pleural pain, the placing of the patient in the upright position, are of this order. So too are specific or other forms of treatment which by reducing fever decrease the metabolism, and hence the carbon dioxide output, and so decrease the amount of ventilation necessary to preserve the normal acid-base ratio. As Hoover (30) has pointed out, anoxemia may or may not be relieved by oxygen administration, but in any event difficulties in carbon dioxide elimination cannot be so relieved, and consequently it seems unlikely that oxygen administration will have any great beneficial effect in the treatment of pneumonia. Several years ago Newburgh, Means, and Porter (31) showed that the respiratory center gradually fails during the course of experimental pneumonia. It has also been shown by Davies, Haldane, and Priestley (32) that resistance to breathing actually causes fatigue of the center. It is possible that this failure was in part due to such fatigue, the fatigue resulting from an incompletely successful respiratory effort to overcome a tendency towards carbonic acidosis.

#### SUMMARY.

Carbon dioxide diagrams (Haggard and Henderson (9)) have been constructed for the blood of a series of hospital patients as a method of studying disturbances in their acid-base equilibrium.

A diabetic with a low level of blood alkali, but with a normal blood reaction, a compensated acidosis in other words, showed a rapid return towards normal with no treatment but fasting and increased water and salt intake.

A nephritic with a decompensated acidosis and a very low blood alkali was rapidly brought to a condition of decompensated alkalosis with a high blood alkali by the therapeutic administration of sodium bicarbonate.

It is suggested that the therapeutic use of alkali in acidosis is probably only indicated in the decompensated variety, and that there it should be controlled carefully and the production of alkalosis avoided.

The diagram obtained in three pneumonia patients suggested that they were suffering from a condition of carbonic acidosis, due perhaps to insufficient pulmonary ventilation.

In two out of three cases of anemia the dissociation curve was found to lie at a higher level than normal. No explanation for this finding was offered.

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## RELATION BETWEEN THE VIRULENCE OF STREPTOCOCCI AND HEMOLYSIN.

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(Received for publication, September 28, 1920.)

Since the original observations on streptolysin by Marmorek (1) in 1902, there has been much discussion concerning the relation between the hemolytic property and the pathogenicity of streptococci. Clinical and laboratory studies have been made, but, due to the complexity of the problem, the opinions arrived at have been contradictory. In 1914, M'Leod (2) reviewed the literature, and although he believed that there was an intimate connection between hemolytic power and virulence, he considered that there had been no solution of the problem which had been generally accepted. To attempt a solution of the problem purely on a clinical basis is obviously impossible on account of the great variations in susceptibility of individuals to infection. This is undoubtedly the reason that observations depending on the course of any infection as an indication of pathogenicity have led to such indefinite and opposed conclusions. If it is possible to establish definite facts in the laboratory under constant environment suitable for the growth of the bacteria, the same methods can probably be applied to the clinical phase of the problem.

The ideal laboratory experiment has been indicated by M'Leod (3). Briefly, it consists in testing the hemolytic power of the streptococcus *in vivo*; there are, however, no trustworthy methods with which this procedure can be accurately carried out. The obvious substitute is to observe the hemolytic titer of virulent strains in the serum of the animal for which they are pathogenic. We attempted to use rabbits in this manner but had great difficulty in obtaining strains which were

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of constant virulence, and, furthermore, it was found that the hemolytic titers of cultures growing in rabbit serum varied considerably. In fact the only medium which gave consistent maximum titers was 20 per cent horse serum broth. Owing to these difficulties we have attempted first to determine the relation between pathogenic and non-pathogenic strains in respect to their lytic power, when they are grown in the serum of an animal for which their virulence has not been especially increased.

#### *Method.*

*Media.*—The character of the media employed is undoubtedly the most important essential for the production of strong hemolysin. Beef infusion broth to which horse serum had been added to 20 per cent of the volume was found to be the most satisfactory for the comparison of various strains. If the broth is made with 2 per cent peptone the titers are quite constant in different experiments with the same streptococcus. To ensure uniformity all the media used in this series were made at the same time from the same lot of beef infusion. They were titrated so that the pH was 7.6 after sterilization, and were distributed in quantities of 80 cc. in 250 cc. Pyrex flasks. The horse serum was obtained from the same horse in each instance. While it was still fresh 20 cc. were added to each flask. The contents of the flasks were then inactivated at 56°C. on 3 successive days and stored on ice until used. In this way it was possible to grow the cultures under conditions in which the available protein substances and the antihemolysins were constant.

*Seeding and Bacterial Counts.*—The second factor which may vary sufficiently to destroy the accuracy of the results is the nature of the seeding. According to De Kruif and Ireland (4) young cultures produce streptolysin more rapidly than those which have ceased to grow actively. Since it had been previously found (5) that, under the conditions which we employed, the period of active growth ceased after about 16 hours, the flasks were seeded with horse serum broth cultures from 14 to 18 hours old. The seeding was controlled by counting a dilution of bacteria in a weak aqueous solution of methylene blue. A Helber counting chamber with a thin cover-glass was used so that the count could be made with either a high power dry lens

or with an oil immersion lens. The chains of bacteria were counted and the average number of individuals in each group was estimated by counting the cocci in a film made from the dilution pipette. It was possible to approximate the number of cocci in each cubic centimeter of the culture used for seeding by this method. On account of the inaccuracy of the bacterial counts each experiment was done in duplicate or triplicate by seeding several flasks so that they contained approximately 1, 5, and 10 million per cc. The flasks were counted a second time before incubation by the dilution and plating method described in a previous article (5), and the growth of the cultures was followed at intervals in a similar manner. It was impossible to count the streptococci with any great degree of accuracy, but by seeding several flasks with varying numbers of cocci, a comparable growth was obtained in at least two. After several experiments it was found that the counts were unnecessary because in the tests in which the flasks were seeded within a fairly close range, the minimum hemolytic dose was the same in each flask of the triplicate experiments.

*Hemolysin Titers.*—Hemolysis was determined with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. The supernatant fluid was set up in a series of tubes with 1 cc. of 5 per cent mouse cells washed and suspended in isotonic salt solution. The smallest quantity of fluid necessary to hemolyze these cells completely without leaving sediment was called the titer of the culture. These determinations were made throughout the experiment, but more frequently during the time that streptolysin production was at a maximum. Since it is impossible to compare titers with different suspensions of red cells, a sufficient quantity was prepared to complete a single experiment. The character of the red cells does not change sufficiently when they are suspended in isotonic salt solution to vitiate results obtained within a period of 16 to 18 hours after the cells are placed in suspension. The titers obtained by these methods were practically constant for each strain.

#### EXPERIMENTAL.

Five strains of beta type streptococci obtained from acute human infections, in some instances from the blood stream and in others from pleural exudates, were used. They conformed to the *Strepto-*

*coccus pyogenes* of Holman (6) and gave a final hydrogen ion concentration of pH 4.9 to 5.2 in glucose broth. Before these strains were used for the present experiments they were stored on blood agar and transplanted at frequent intervals during a period of several months, in order that they might lose their original virulence for animals.

At the beginning of the experimental work the invasive power of each strain was determined on mice of approximately the same weight. They were then passed through mice by intraperitoneal injection and obtained in pure culture from the heart's blood. The doses were regulated so that the animals died within 24 hours. After each passage the strain was transferred to rabbit blood agar in the second subculture. When each streptococcus was sufficiently invasive, the avirulent and virulent forms were transplanted from the blood agar tubes into horse serum broth, then, after an interval of 14 to 18 hours, the trial flasks of bouillon were seeded with the necessary quantities of these cultures. In this way the streptolysin production was determined with actively growing cocci which were accustomed to the media in which the test was made. After the hemolysin tests, the virulence of the streptococci was again determined on mice with the corresponding subcultures from the stock media.

*Experiment 1.*—Strain J, which had been in stock culture for 2 months was passed through twenty mice. At the end of the passages the original culture and the virulent mouse strain were grown in horse serum broth and seeded into 100 cc. flasks of media. The seedings were made so that there were three flasks for each strain containing approximately 1, 5, and 10 million cocci per cc. The bacterial counts (Text-fig. 1) and the hemolysins were determined at frequent intervals until after the maximum streptolysin production had occurred. The pathogenicity for mice was determined immediately after the experiment, with a 24 hour plain broth culture of Strains J(0)5 and J(20)5.<sup>1</sup> The results of the tests are shown in Tables I and II.

*Experiments 2 to 5.*—The previous experiment was repeated with Strains K, M, R, and S. Only two seedings were used for each strain, however, one of 1 and one of 10 million cocci per cc. Table III summarizes the maximum titers, the hour at which they occurred during the growth of the culture, and the pathogenicity of the various strains for mice.

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<sup>1</sup> The strains have been indicated by letters, the number of passages by a figure in parentheses, and the subculture by the second numeral.



TABLE I.  
*Streptolysin Production by Strain J.*

Interval.	1 million.*		5 million.		10 million.	
	Count.	Titer.†	Count.	Titer.	Count.	Titer.
Strain J(0)5, not passed through animals.						
hrs.	cc.		cc.		cc.	
0	1.1 (10 <sup>6</sup> )‡	0.0	5.5 (10 <sup>6</sup> )	0.0	1.1 (10 <sup>7</sup> )	0.0
3½	5.0 (10 <sup>7</sup> )	0.0	4.7 (10 <sup>8</sup> )	0.0	6.0 (10 <sup>8</sup> )	0.6
6		0.02		0.04		0.04
7½	1.0 (10 <sup>9</sup> )		1.3 (10 <sup>9</sup> )		1.0 (10 <sup>9</sup> )	
8		0.06		0.08		0.08
10		0.06		0.2		0.15
12	8.0 (10 <sup>8</sup> )		7.5 (10 <sup>8</sup> )		2.0 (10 <sup>9</sup> )	
Strain J(20)5, after passage through animals.						
0	2.4 (10 <sup>6</sup> )	0.0	1.2 (10 <sup>7</sup> )	0.0	2.4 (10 <sup>7</sup> )	0.0
4	1.5 (10 <sup>7</sup> )	0.0	7.3 (10 <sup>7</sup> )	0.0	5.6 (10 <sup>8</sup> )	0.0
5½		0.4		0.06		0.08
7		0.4		0.04		0.04
8	6.5 (10 <sup>8</sup> )		1.6 (10 <sup>9</sup> )		7.8 (10 <sup>8</sup> )	
9		0.04		0.06		0.06
11	9.8 (10 <sup>8</sup> )	0.04	7.0 (10 <sup>8</sup> )		8.1 (10 <sup>8</sup> )	
12		0.06				

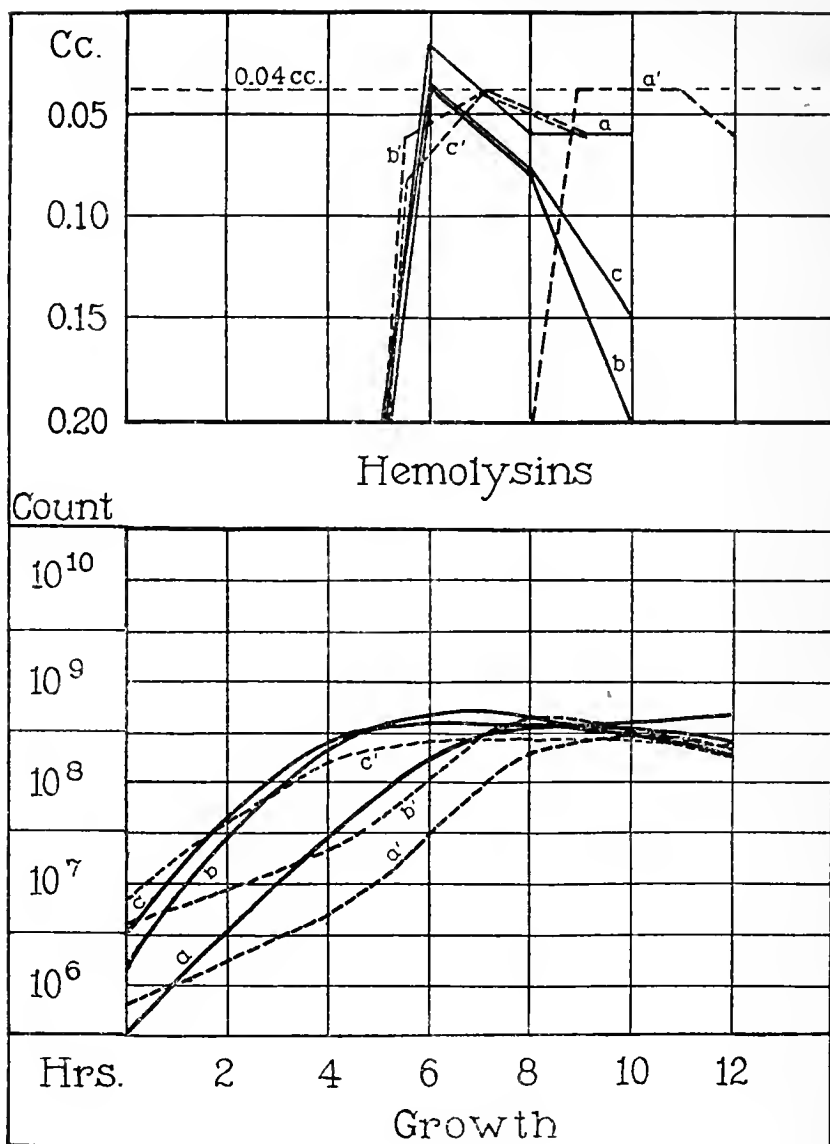
\* The counts designated in millions were calculated from the direct counts used for seeding. The others are expressed in powers of ten.

† The titers are the smallest amounts of the supernatant fluid which completely hemolyzed 1 cc. of the cell suspension after 1 hour at 37°C.

‡ All counts in the columns were made from dilutions and plates.

TABLE II.  
*Pathogenicity of Strain J for Mice.*

Strain.	Quantity of culture.	Length of time animal survived.
	cc.	hrs.
No. J(0)5, not passed through animals.	1.4	18
	1.2	18
	1.0	(Recovered.)
	0.8	"
	0.6	"
No. J(20)5, after passage through animals.	0.2	17
	0.1	17
	0.08	17
	0.06	18
	0.02	(Recovered.)



----- Strain J (20) 5.  
 ————— Strain J (0) 5.

TEXT-FIG. 1. Comparison of the growth and of hemolysin production by Strain J before and after mouse passage. The counts are charted logarithmically. Curves *a* and *a'* give the results obtained with original seedings of 1 million, Curves *b* and *b'* with original seedings of 5 million, and Curves *c* and *c'* with original seedings of 10 million.

TABLE III.

*Streptolysin Production and Pathogenicity for Mice of Strains J, K, M, R, and S.*

Strain No.	Seeding.	Titer.	Time at which maximum titer occurred.	Pathogenicity.*
	<i>million†</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>
J(0)5	1	0.02	6	1.2
	5	0.04	6	
	10	0.04	6	
“(20)5	1	0.04	9	0.06
	5	0.04	7	
	10	0.04	7	
K(0)2	1	0.04	7½	1.0
	10	0.04	7½	
“(17)2	1	0.04	12	0.1
	10	0.04	12	
M(0)4	1	0.04	10	‡
	10	0.02	8	
“(19)4	1	0.02	8	0.5
	10	0.02	8	
R(0)4	1	0.04	8	0.4
	10	0.01	8	
“(19)4	1	0.02	8	0.03
	10	0.04	8	
S(0)2	1	0.04	6½	1.0
	10	0.04	6½	
“(17)2	1	0.04	10	0.2
	10	0.04	8	

\* The pathogenicity is indicated by the smallest quantity of a 24 hour broth culture fatal to mice in 24 hours.

† Based on direct counts and controlled by dilution and plating.

‡ Animals survived doses of 5 to 10 cc. of centrifuged culture.

## DISCUSSION.

From the results of these experiments it appears that both the virulent and avirulent types of each strain produce approximately the same maximum titer at some time during the growth of the cultures. Grown under the conditions previously described the strains have yielded titers in the majority of instances of 0.04 cc.

It is not to be expected that various experiments can be compared in this respect, when they are carried out on different days with different red cell suspensions. The quantities of culture fluid required to hemolyze the cell suspension in any one experiment are fairly consistent, with the exception of one in which Strain R was used in seeding. A certain degree of variation can be anticipated because of the difficulty experienced in reading the last completely hemolyzed tube in a series graded in 0.01 cc. With two of the five strains, M and R, the peaks of the hemolysin curves were simultaneous in the cultures of both the virulent and less virulent forms. With Strains J, K, and S the former lagged from 1 to 4½ hours. This lag was apparently due to the fact that the more virulent strains grew more slowly during the first hours of incubation. This fact was evident from the amount of cloudiness in the flasks as well as from the plate counts. In the two instances in which the highest points of the hemolysin curves were reached simultaneously, the cultures of the strains which had not been passed through animals were moderately hemolytic for some time before the other flasks had any destructive action on red cells.

#### CONCLUSION.

Strains of streptococci whose virulence has been increased for any one species of animal do not produce greater concentrations of hemolysin than the original strain. Furthermore, there is a tendency for the original culture to grow more rapidly than the more pathogenic form, and to reach the height of hemolysin production at an earlier stage during the growth of the culture. These conclusions can probably be applied only to experiments in which the serum used in the media is from some species not employed for the animal passages.

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QUANTITATIVE DISTRIBUTION OF PARTICULATE MATERIAL (MANGANESE DIOXIDE) ADMINISTERED INTRAVENOUSLY TO THE DOG, RABBIT, GUINEA PIG, RAT, CHICKEN, AND TURTLE.

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(Received for publication, November 11 1920.)

The convenient method for manganese determination developed by Reiman and Minot (1) has been utilized by Drinker and Shaw (2) in a series of observations upon the distribution of finely divided manganese dioxide introduced intravenously in the cat. A discussion of the extensive literature upon the intravenous injection of insoluble foreign particles, together with a detailed description of the technique employed in their experiments, will be found in the latter paper. Since plans for future work have made necessary the use of other laboratory animals than the cat, and since the possibility of differences between species in the ability to clear the blood stream of foreign material has been insufficiently investigated, a similar study to that made by Drinker and Shaw (2) with the cat was undertaken with other common laboratory animals. The results are reported in the present paper.

EXPERIMENTAL.

The suspensions of manganese dioxide employed contained no particles above 1 micron in size, and on analysis varied in manganese content from 0.140 to 0.903 mg. of manganese per cc. The number of particles varied between 2.5 and 5 billion per mg. of manganese. Tissues to be analyzed were taken from the body immediately after the animal had been bled to death, and the results are expressed in milligrams of manganese per 100 gm. of wet tissue. The results of Bertrand and Medigreceanu (3), with work of Reiman and Minot (1) and with analyses made by ourselves, indicate that the normal

manganese content of the tissues reported in this paper is negligibly small. The amounts shown in Table I are of no significance in view of the comparatively large injections made—large enough to assure detection of manganese in any organs in which manganese dioxide particles lodge.

In all instances injections of manganese dioxide suspensions were made under urethane anesthesia, and in the dog and rabbit records of blood pressure and respiration show, as in the cat (2), that no toxic

TABLE I.

*Average Normal Manganese Content per 100 Gm. of Wet Tissue.*

Organ.	Amount of manganese per 100 gm. of wet tissue.						
	Fifteen cats.	Eight dogs.	Eight rabbits.	Two guinea pigs.	Two rats.	Two chickens.	Two turtles.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Liver.....	0.341	0.238	0.178	0.371	0.326	0.311	0.119
Spleen.....	0.069	0.022	0.054				
Brain.....	0.035	0.044	0.062				
Stomach.....	0.046	0.043	0.059				
Small intestine...	0.044	0.028	0.068	0.137*	0.099*	0.051*	0.061*
Colon.....	0.086	0.080	0.099				
Cecum and appendix.....			0.043				
Kidney.....	0.136	0.087	0.147	0.000	0.110	0.124	
Lung.....	0.031	0.023	0.036	0.000	0.000	0.000	0.000
Muscle.....	0.025	0.020	0.022	0.043	0.000	0.030	0.016
Bone.....			0.101				
Urine.....	0.000	0.000	0.000				
Blood.....	0.000	0.000	0.000				

\* Entire intestinal tract.

symptoms attend such injections. While records were omitted during the injection of the smaller animals no unfavorable symptoms were noted.

The injected animals were killed by bleeding to death from the carotid arteries at the end of 1 hour. Organs for analysis were at once removed, weighed wet, and the manganese content was recorded in milligrams per 100 gm. of tissue. Drinker and Shaw (2) found for the cat that the liver, lungs, and spleen are the points of maximum lodgment, and our observations indicate that this is also true for

other animals. Table II shows the small amounts removed by tissues exclusive of the liver, lungs, and spleen in the rabbit. Table III presents the percentages of injected manganese deposited in the liver, lungs, and spleen of the animals employed. A column is also included, which obviously cannot be completely accurate, to represent the traces found in the blood, and a final column represents the percentage of injected material deposited in other organs than those

TABLE II.

*Distribution of Manganese in Tissues Exclusive of the Liver, Lungs, and Spleen in the Rabbit after the Intravenous Injection of Manganese Dioxide.*

Weight 2.6 kilos. Mar. 9, 1920. Injection of 13.6 cc. of manganese dioxide suspension containing 19.3 mg. of manganese per 100 cc. Total injection 2.62 mg. of manganese.

Organ.	Amount of man- ganesee recovered.	Per cent of injected manganese recovered.
	mg.	
Brain.....	0.000	0.00
Stomach.....	0.000	0.00
Small intestine.....	0.031	1.2
Colon and rectum.....	0.013	0.5
Cecum and appendix.....	0.000	0.00
Kidney.....	0.007	0.3
Bone.....	0.004*	2.2†
Heart.....	0.002	0.1
Blood.....	0.022	0.7‡
Total.....	0.079	5.0

\* Tibia.

† Bone weight of body calculated at 5 per cent of body weight.

‡ Blood weight of body calculated at 7 per cent of body weight.

chiefly concerned in removing foreign particles from the blood stream. The latter amount varies between 19 per cent in one guinea pig and 2 per cent in one rat. It is impossible to foretell why such variations occur and where the manganese will be found. In the rabbit even during the 1st hour following injection there is a variable degree of excretion in the bile. Some of this manganese is thus included in analyses of the duodenum and jejunum. The intestinal wall of all

the animals studied shows small and very variable amounts of deposition. The same is true of muscle and kidney.

Text-fig. 1 is a graphic representation of the data from Table III. The most striking feature of the figure is that the lungs of the cats contain practically 50 per cent of the manganese 1 hour after injection, while the lungs of all the other animals contain minute amounts, Dog 1 with 14 per cent being the single exception to this.

TABLE III.

*Distribution of Manganese 1 Hour after the Intravenous Injection of Manganese Dioxide.*

Animal.	Amount of manganese injected.	Per cent of injected manganese recovered.				
		In liver.	In lungs.	In spleen.	In blood.*	In other organs.
Average for twenty cats.....	mg.	38.3	47.0	4.7	0.0	10.0
Rabbit 1.....	7.98	81.0	1.2	0.8	0.6	16.5
“ 2.....	6.18	81.0	0.2	2.5	0.7	15.4
“ 3.....	2.71	85.6	0.52	0.55	0.0	13.3
Dog 1.....	83.5	64.0	14.0	2.0	3.9	16.1
“ 2.....	45.2	83.0	3.7	2.1	2.1	9.1
“ 3.....	67.7	79.0	2.0	4.0	0.9	14.1
Guinea Pig 1.....	2.03	76.0	2.1	1.7	1.2	19.0
“ “ 2.....	2.34	85.0	0.8	1.4	0.3	12.5
Rat 1.....	1.34	94.0	0.5	1.9	0.0	3.6
“ 2.....	0.83	98.0	0.6	0.0	0.0	2.0
Chicken 1.....	4.36	84.0	0.0	1.7	0.0	14.3
“ 2.....	2.82	84.0	0.4	1.7	0.0	13.9
Turtle 1.....	1.13	94.0	3.0	No analysis.	0.0	3.0
“ 2.....	1.0	88.0	7.5	“ “	0.0	4.5

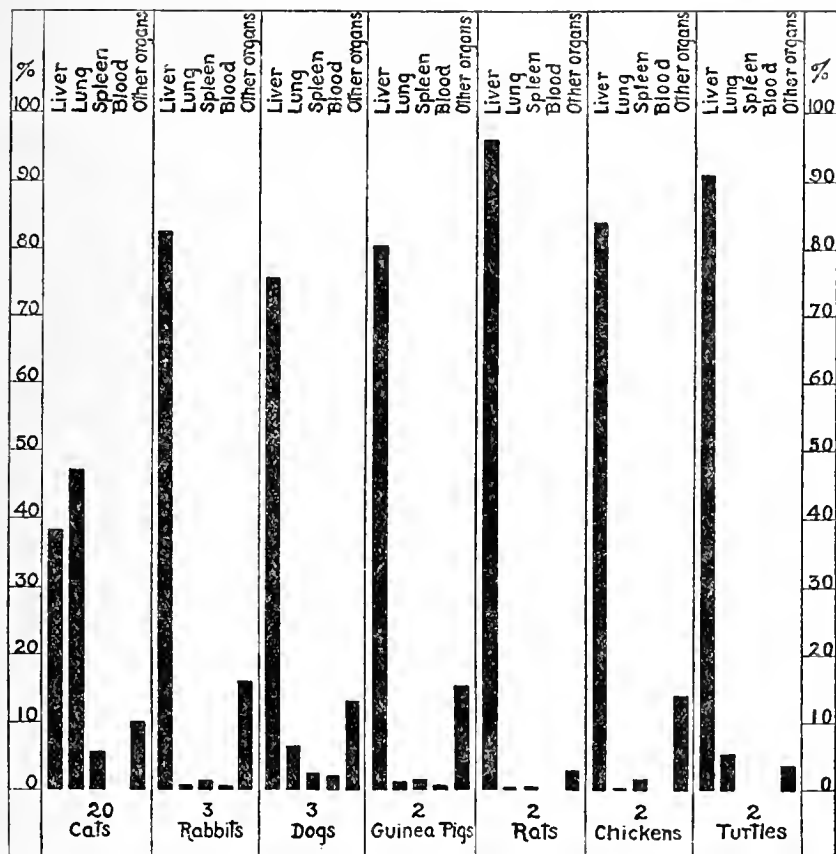
\* Blood weight calculated at 7 per cent of body weight.

In the animals whose lungs removed less of the suspension from the circulating blood the liver removed more manganese so that the amount scattered among the other tissues was very little greater or was even less than in the cats.

In Text-fig. 2 the distribution of manganese dioxide 12 hours after injection in the cat is shown. At this period the animal had lost the high lung concentration which was observed 1 hour after injection. The material had accumulated in the liver, from which it slowly

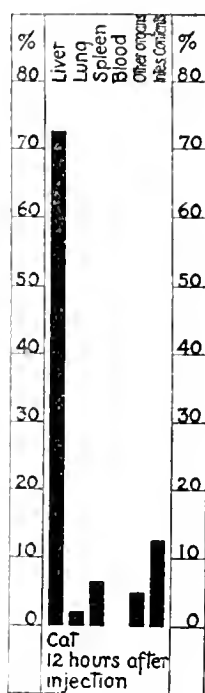


escaped through the bile. With the rabbit and guinea pig killed 15 minutes after injection, no difference was found in distribution of manganese dioxide from that shown in Text-fig. 1. These animals,



TEXT-FIG. 1. Distribution of manganese in the organs of animals 1 hour after the intravenous injection of manganese dioxide.

therefore, unlike the cat do not go through the stage of deposition in the lungs and final collection in the liver, but the foreign material is at once localized where its removal from the body is best accomplished.



TEXT-FIG. 2. Distribution of manganese in the organs of a cat 12 hours after the intravenous injection of manganese dioxide.

#### DISCUSSION AND CONCLUSIONS.

The experiments presented may be contrasted with but little other work with injected particles, since previous experimentation has lacked any quantitative basis. Voigt (4) has published a series of papers on the distribution of colloidal silver and colloidal silver iodide. He determined the distribution both chemically and microscopically. His suspensions differed from ours in several ways. In the first place, the individual particles of his suspensions were much finer, having an average diameter of 15 to 20 micromicrons in contrast to the 0.6 to 1 micron diameter of the manganese dioxide particles used in the present study. Also, the concentration of Voigt's suspensions was far greater, ranging from 10 to 50 per cent in contrast to 0.015 to 0.09 per cent in our work. Furthermore, some of his suspensions must have shown agglutination, since he found emboli in the lungs of rabbits. In spite of these differences, three rabbits, killed at a longer interval following injection than the animals in our experiments, displayed 0.2, 5.1, and 3.7 per cent of the injected material in the lungs, out of a total of 38, 89, and 63 per cent recovered on analysis of all tissues.

In the same experiments the livers contained respectively 35, 80.7, and 51.8 per cent of the material injected. These rather rough results agree with our own and confirm the indication that in the rabbit the lungs play a very minor part in clearing the blood of foreign particles.

More recently Duhamel (5) has published a brief note upon injections into rabbits of colloidal platinum, selenium, mercury, copper, and iron. On chemical analysis he found a large proportion of the injected material in the liver. Again the individual particles were very small but the function of the liver in removing them from the circulation is obvious.

It has been recognized for many years that soluble salts of the heavy metals are deposited from the blood in the liver, and that this deposition plays an important part in the removal of such salts from the body. Clearly, the organ operates with an equal degree of efficacy in the case of particles up to 1 micron in diameter, and probably would do so with even larger sizes, though as yet we have no data upon the subject.

As Drinker and Shaw (2) have discussed much of the bacteriological work relating to removal of organisms from the blood stream, it is only necessary here to mention such data as display differences between the lung element in the reaction. Bartlett and Ozaki (6), using histological methods for detection, found larger numbers of staphylococci in the lungs of dogs and rabbits killed 1 to 30 minutes after injection than in other organs. By the end of an hour fewer bacteria were detected in the lungs than in the liver and spleen. Arima (7) found few bacteria (*Staphylococcus aureus*, *B. coli*, and *B. typhosus*) in the lungs of rabbits 1 hour after injection. Kyes (8) found few pneumococci in the lungs of pigeons. These observations, with the exception of those of Bartlett and Ozaki (6), agree well with our findings in the case of manganese dioxide particles. It is only possible to express a rough opinion as to quantities of material on the basis of histological examination, and we are thus inclined to believe that the atypical results of Bartlett and Ozaki (6) represent an error in judgment and do not affect the facts of the case.

The most important and conclusive work with bacteria is that of Hopkins and Parker (9). They found a marked difference in distribution of streptococci injected intravenously into cats and rabbits. Using comparatively accurate cultural methods they found the following distribution of streptococci 10 minutes after injection.

The result is in conformity with that obtained in the present study for manganese dioxide particles of approximately the same size as streptococci. It is not known why the cat and the rabbit display these differences, but fortunately the cat is unique among the labo-

Organ.	No. of streptococci per 0.1 gm. of tissue	
	In cat.	In rabbit.
Lung.....	315,000	41,000
Liver.....	34,000	104,000
Spleen.....	18,000	120,000
Kidney.....	100	0
Bone marrow.....	500	
Psoas.....	8	1,500
Pectoralis.....	27	
Blood.....	26	

ratory animals tested and is seldom used for bacteriological work.

#### SUMMARY.

The distribution of manganese dioxide particles 1 hour following intravenous injection in cats, dogs, rabbits, guinea pigs, rats, chickens, and turtles is described. This distribution is remarkably constant for all the animals tested, except the cat, in which the injected material is practically equally divided between the lungs and liver. In the other animals the liver performs the main share of the work, and in the cat it has been shown that the liver after 12 hours accumulates the manganese which was formerly deposited in the lungs.

The results are in harmony with experiments in which bacterial suspensions are employed for injection and confirm the suggestion previously made (2) that in the first handling of foreign particulate material the animal behaves similarly whether protein or inorganic injections are used.

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## EXPERIMENTAL STUDIES ON YELLOW FEVER IN NORTHERN PERU.

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(Received for publication, August 7, 1920.)

*Leptospira icteroides*, first isolated from certain cases of yellow fever in Guayaquil,<sup>1</sup> and later from a case of this disease in Merida,<sup>2</sup> had assumed such significance in the study of the etiology of yellow fever as to make further investigations advisable. Peru, which has had many visitations of yellow fever, was again invaded in June, 1919, in the province of Piura, the northernmost region, bordering on Ecuador. From this invasion a small epidemic arose which had not entirely disappeared in May, 1920. During the outbreak the following towns were affected: Sechura, Morropon, Tambogrande, Chulucanas, Piura (500 cases among the 10,000 inhabitants) in 1919, and Payta (108 cases among 3,000 inhabitants) in 1920. The mortality was estimated to have been about 10 per cent, which is considerably lower than was the case with yellow fever in Guayaquil and Merida, where it was about 50 per cent. An expedition to Peru was therefore undertaken.<sup>3</sup> The present report deals with the results of bacteriological studies at Payta, Piura, and Morropon extending over a period of 3 months, March, April, and May, 1920.

When one of us reached Peru (March 1, 1920) Payta was the only town where the epidemic of yellow fever was still in progress; the last case occurred there on April 16. The first experiments were

<sup>1</sup> Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

<sup>2</sup> Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, 601, 627.

<sup>3</sup> This expedition was undertaken under the auspices of the International Health Board of The Rockefeller Foundation, and The Rockefeller Institute for Medical Research. We wish to thank the federal and local authorities in Peru for their courtesy and cooperation in this work.

carried out in Payta. In April an epidemic was reported at an inland town, Morropon, and a trip was made to that place to secure material for further studies to be carried out in Piura, where better laboratory facilities were available.

### *Studies in Payta.*

Payta, a town of about 3,000 inhabitants, is the principal port in northern Peru. It consists of a cluster of bamboo huts on a strip of sandy shore. Rain is rare, and water is very scarce. The water supply comes from a river about 7 kilometers from the town, but the amount is hardly sufficient for ordinary daily needs.

A provisional laboratory was set up in Payta in a small bungalow consisting of three rooms. One room served as a laboratory, another as an animal room, and the third as a sleeping room. The laboratory supplies brought from The Rockefeller Institute had been reesterilized in the laboratory of the Municipal Institute of Hygiene in Lima, as there were no facilities for steam or hot air sterilization at Payta. The rabbit serum used in the culture media was brought from New York and as a result of the long voyage in a tropical climate a precipitate had appeared in it. The guinea pigs, also brought from New York, had suffered severely, and about two-thirds of them had succumbed within 2 weeks of their arrival in Payta. The feed for the guinea pigs was scarce, so that only the larger and hardier animals survived. This was unfortunate, as the larger animals are less suitable for initial inoculation; however, a certain number of native guinea pigs was procured. A very serious circumstance was the fact that, owing to the lack of electric current, the dark-field microscope could not be used. Moreover, the effort to obtain Giemsa preparations of the blood were unsuccessful because of the quality of the water. Since most yellow fever patients were treated by their physicians at their own homes it was not always easy to secure consent to obtain blood for inoculation, and it was practically impossible to obtain blood twice from the same patient. Finally, the cultures of *Leptospira icteroides* brought to Payta from Merida did not survive the journey.

Under the adverse conditions and the lack of laboratory facilities, the bacteriological work was confined to cultivation and animal

transmission with such samples of blood as could be obtained, the object being to infect guinea pigs and to produce the characteristic symptoms and lesions in these animals. In all, nine cases of yellow fever were studied. Guinea pigs brought from New York were inoculated from seven and native guinea pigs from two of the cases.

*Case 1 (Fatal).*—B. Onset Mar. 10, 1920. Mar. 12. Temperature 39°C.; pulse 110; albuminuria. Mar. 14. Temperature 38.5°; pulse 88; albumin 1 gm. per 1,000 cc. Mar. 16. Delirious; black vomit; anuria. Mar. 17. Died.

Mar. 12 (3rd day of illness). Blood taken and cultures made (nine tubes). Mar. 16. 1 cc. of citrated blood (kept in the ice box for 4 days) was inoculated intraperitoneally into Guinea Pig 1 and 2 cc. into Guinea Pig 2. The culture tubes, which had stood at room temperature for 4 days, appeared free from contamination, and three guinea pigs were inoculated with material from Tubes 1 to 4 and three with material from Tubes 5 to 9. Some of the culture material was left standing until Mar. 23, when it was inoculated into three guinea pigs.

The two guinea pigs inoculated with blood 4 days old showed no fever. A few old hemorrhagic areas were found in the lungs of Guinea Pig 2 when it was killed on the 15th day. Of six guinea pigs inoculated with culture material 4 days old, three had fever on the 6th day and showed petechial lung hemorrhages when killed on the 15th day. The three remaining animals either suffered from an intercurrent infection (pneumonia) or escaped any obvious infection.

Of the three guinea pigs inoculated with the 11 day culture material, one (Guinea Pig 43) showed petechial hemorrhages in the lungs when killed on the 15th day. The other two showed on autopsy no leptospira lesions but enlarged spleen and pulmonary congestion, which were taken as evidence of secondary infection.

The findings described show that in no instance was a fatal infection by *Leptospira icteroides* induced, but they raise the question whether the characteristic hemorrhagic areas in the lungs in Guinea Pigs 2, 3, 5, 8, and 43 did not indicate a mild infection with this organism.

*Case 2 (Moderate; Recovered).*—C. Onset Mar. 13, 1920. Moderate case; course typical. Mar. 20. Recovered.

Mar. 14 (2nd day of illness). Blood taken and used wholly for making cultures. Three guinea pigs were injected with a 3 day culture, three with a 4 day culture, and three with a 9 day culture.

Of this series almost all, except Guinea Pig 14, which received a 3 day culture, and No. 19, which received a 4 day culture, died of intercurrent infections (pneumonia, paratyphoid, cocci), while two remained well. Guinea Pig 14 showed on the 5th day a temperature of 39.7°C., and on the 6th 39.6°, while at autopsy the lungs showed several hemorrhagic areas. Guinea Pig 19 showed on

the 7th day a temperature of 39.5° and on the 8th and 9th days 39.8°. It was killed on the 10th day for examination and transfers. Few small petechiæ in the lungs and minute points of hemorrhage in the right kidney. The blood and organ emulsions from this animal were inoculated on Mar. 28 into two guinea pigs, both of which soon returned to normal.

The experiments on Case 2 are suggestive and lead to the tentative conclusion that in two at least of the nine guinea pigs inoculated with culture materials prepared with blood drawn on the 2nd day a mild infection with *Leptospira icteroides* was induced. It is possible that Guinea Pig 19, if allowed to live longer, might have developed a typical form of the *icteroides* infection, as, when killed on the 10th day, definite lesions were present in the lungs and kidney. The failure to transfer the infection from this animal into two others is not conclusive, as in the early transfers a larger number of guinea pigs should be employed, because of the resistance to infection which certain guinea pigs usually exhibit.

Case 3 (recovered) gave similar results with blood drawn on the 3rd day of illness.

Two guinea pigs were inoculated with 2 cc. of blood from Case 4 (fatal; blood drawn on 2nd day of illness) soon after it was drawn, and two with the same amount of blood from Case 5 (recovered; blood drawn on 3rd day of illness). Cultures made with blood from each of these patients were left at room temperature for 4, 7, and 10 days and then inoculated into eight guinea pigs (Mar. 19, 22, and 26). Some of the animals inoculated with material from Cases 4 and 5 had definite febrile reactions and showed at autopsy lung lesions suggestive of a mild leptospira infection, but there was no fatal infection with typical jaundice.

With Case 6 (recovered; blood drawn on Mar. 19, 2nd day of disease) and Case 7 (recovered; blood drawn on Mar. 20, 3rd day of disease), the blood was drawn into citrate serum agar mixed in equal parts, and 3 cc. of the mixture were inoculated into each of two guinea pigs. Cultures made in the usual way were allowed to stand at room temperature for 3 to 7 days, and two sets of six guinea pigs were inoculated with this material. The results with Cases 6 and 7, both with blood and cultures, were unsatisfactory. The majority of the guinea pigs showed irregular febrile reactions, and from these animals, owing to the scarcity of guinea pigs, no transfers were made. Some of them, when killed later, were found to have hemorrhagic areas in the lungs, some showed indications of secondary infection (enlarged spleen), while others showed no lesions. In no instance was there a typical fatal leptospira infection.

Two more cases were studied before the epidemic in Payta subsided, Case 8 (recovered; blood drawn on 3rd day of illness, Mar. 29) and Case 9 (recovered;



blood drawn on Apr. 1, 2nd day of illness). In view of the failure to secure a definite transmission with fatal outcome with the larger guinea pigs still alive from the lot brought from New York, we decided to test native guinea pigs. For this purpose six native and two American guinea pigs, weighing 600 gm., were inoculated with a 7 day culture of the blood of Case 8 and five native guinea pigs with the 10 day culture from Case 9. None of the animals developed a typical fatal leptospira infection, although some undoubtedly had a mild infection, since lung lesions were found at autopsy and in two instances there was a suggestion of jaundice. It is interesting to note here that later experiments demonstrated that the native guinea pigs possess a greater resistance to the *icteroides* infection than the domesticated variety brought from the United States.

It is obvious that in the transmission experiments just described as having been carried out at Payta, no typical instance of fatal infection with *Leptospira icteroides* was obtained, and in no instance was the leptospira observed under the microscope. As the dark-field microscope was not available and no proper Giemsa staining could be secured, the latter circumstance is without value.

On the other hand, certain positive results were obtained in inoculated guinea pigs which led to the belief that a mild form of *Leptospira icteroides* infection had in some instances been induced; *i.e.*, rise of temperature after the period of incubation common in this infection (3 to 5 days) and at autopsy definite hemorrhagic areas in the lungs and in one instance in both lungs and kidney, with occasionally a suggestion of jaundice. The failures to obtain more pronounced results are not difficult to account for. As stated above, almost all the guinea pigs of the most favorable age and weight shipped from New York succumbed *en route* or soon after arriving at Payta. Those remaining were so few in number that they were used sparingly; hence fewer were injected with given samples of blood or cultures than would ordinarily have been employed. The rabbit serum which is essential to successful cultivation of the leptospira had undergone changes with the formation of a precipitate, and the reaction became so alkaline as to prevent a growth of *Leptospira icteroides* to any extent. And yet a degree of success, which was confirmed by subsequent results, was, we believe, achieved.

*Studies in Piura.*

The epidemic having subsided in Payta, the laboratory was removed to Piura, where sterilizing facilities and adequate water were available. A laboratory was set up, through the cooperation of the government, in the Belan Hospital. A detached building was used for animal quarters and feed was also more plentiful.

By the time the small laboratory had been started cases of yellow fever were reported in Morropon, a small town of 2,000 inhabitants in the foothills of the Andes. The distance from Piura was about 65 miles, a desert separating the two places. Arrangements were made at once to investigate the cases.<sup>4</sup> As the journey was made on horseback it was obviously out of the question to transport experimental animals, etc., by this means through a tropical region; hence it was decided to rely entirely on cultures. Fresh rabbit serum was obtained from local rabbits, and in order to guard against adverse changes in the culture media the component parts, consisting of serum, and 0.3 per cent semisolid agar, were carried separately.

The cultures were made by drawing the blood from an arm vein of the patients directly into the tube of semisolid agar, the rabbit serum then being added in a proportion of 1:5. The whole was thoroughly mixed and covered with a layer of liquid paraffin, and the tubes were carefully capped with tin-foil and carried back to Piura.

On arrival at Morropon it was ascertained that cases of yellow fever had been occurring for some time, and the epidemic was regarded as declining; however, by making house to house visits several cases diagnosed as yellow fever by Dr. Caballero were found on April 23. Between April 24 and 27 cultures with the blood were made from six cases, one of which proved later not to have been yellow fever. The remaining five cases pursued a clinical course which left no doubt as to their yellow fever nature. Because of the illness of one member of the party, the work at Morropon was suspended on April 28.

<sup>4</sup> The journey on horseback from Piura to Morropon usually takes 1½ days. Our party consisted of Dr. Enrico Caballero, the government expert stationed at Catacaos, who showed us every courtesy, Mr. John Mitchell, a sanitary engineer, and Dr. Kligler. The expedition started from Piura on April 21 and arrived at its destination on April 22.

The party returned with the cultures to Piura, reaching there on May 3. At the same time the stock of guinea pigs had been renewed, 300 young, healthy animals having been brought from The Rockefeller Institute. Moreover, because of the lack of electric lighting facilities which had made it impossible to use the dark-field microscope, a storage battery suited to that instrument had also been brought from New York.

Very few of the tubes showed contamination, the blood still appearing bright red in the upper zone of the media. The cultures were inoculated into guinea pigs on May 6, or 9 to 12 days after they had been set up in Morropon. The inoculation procedure was identical with that employed in Merida<sup>2</sup>, the upper portions of several selected tubes of culture from a case being pooled and the mixture inoculated intraperitoneally into six young normal guinea pigs.

Dark-field examination of the culture tubes undertaken the next day (May 7, 10 to 13 days after they were made) revealed the presence of active leptospiras in the cultures from three of the five cases. They were few in number and required careful search in some instances. In some tubes no leptospira was detected. As the details of the experiments show, the inoculation of cultures from four of the five cases induced typical fatal infections in some animals, other animals showing only a mild infection or escaping infection altogether.

*Case 10 (Severe; Recovered).*—C. M., male, age 16 years; born in Salitral; resident of Morropon. Onset Apr. 22, 1920, 7 p.m. Headache; backache; pains in muscles; nausea; no vomiting. Apr. 23. Temperature 39.6°C.; pulse 106. Apr. 24. Temperature 39.9°; pulse 100; albumin +. Apr. 25. Temperature 39°; pulse 90; albumin ++; nausea. Apr. 26. Temperature 37.4°; pulse 90; albumin ++; black vomit. Apr. 27. Temperature 37.6°; pulse 78; albumin ++; epigastric pain; epistaxis; urine increasing towards normal amount; jaundice. Apr. 28. Temperature 37°; pulse 76; recovering.

Blood was taken on the 2nd day of illness at 11 a.m. Cultures examined after 12 days contained living leptospiras. Six guinea pigs (Nos. 13 to 18) were inoculated with material from Tubes 1 and 2 on May 6, with positive transmission in all.

*Guinea Pig 13.*—Temperature 39.2° on the 5th day. Died on the 6th day.

*Autopsy.*—Epistaxis; subcutaneous petechiæ; marked hemorrhages in lungs and gastric mucosa; jaundice slight.

The emulsions of kidney and liver were inoculated into three guinea pigs (Nos. 39 (Chart 1), 40, and 41), all of which developed typical fatal infection, dying

7, 14, and 15 days after inoculation. The leptospiras were found and successful subcultures made.

*Guinea Pig 14.*—Killed, when moribund, to obtain infective material for therapeutic experiments to be described in the following paper.<sup>5</sup>

*Autopsy.*—Numerous petechiæ in lungs; hemorrhage and blood in stomach; liver slightly degenerated; kidneys congested; spleen normal.

*Guinea Pig 15.*—Killed for transfer 4 days after inoculation, at first rise of temperature to 39.4°C.

*Autopsy.*—No lesions were noted, but all three of the guinea pigs (Nos. 36, 37, and 38) inoculated with blood and emulsions of liver and kidneys succumbed with typical infection.

*Guinea Pigs 16 and 18.*—Developed characteristic infection. When jaundice appeared they were utilized for testing the curative effect of the anti-*icteroides* immune serum brought from The Rockefeller Institute, as will be described in the following paper.<sup>5</sup>

With this case leptospiras were found in the initial culture, with which a typical infection was induced in guinea pigs, and further transfer from animal to animal was accomplished. Pure cultures of the leptospira were in turn recovered from the infected guinea pigs.

*Case 11 (Mild; Recovered).*—O. V., male, age 18 years; native of Morropon. Onset in afternoon of Apr. 21, 1920. Chills; headache; backache; fever. First seen morning of Apr. 24. Temperature 40°C.; pulse 100; albumin +. Apr. 25. Temperature 38.5°; pulse 70; albumin ++; nausea, but no vomiting. Apr. 26. Temperature 38.5°; pulse 66; albumin ++. Apr. 27. Temperature 37.2°; pulse 58; albumin +; abundant urine. Apr. 29. Temperature 36.6°; pulse 50; mild jaundice; recovering.

Apr. 24 (3rd day of illness). Blood was drawn. The dark-field examination of cultures 13 days after they were made failed to reveal any leptospira, owing to accidental contamination of the tubes while they were being handled the previous day for animal inoculation.

May 6. Six guinea pigs (Nos. 25 to 30) inoculated with culture, then 12 days old. Three of these (Nos. 25, 26 (Chart 2), and 29) developed typical severe infections, while the remaining three showed no perceptible symptoms. When killed for examination, however, all showed some hemorrhagic areas in the lungs, indications of a mild infection. It is interesting to note the different results with the same culture material, due to variations in individual susceptibility of the guinea pig to *Leptospira icteroides*. The symptoms and lesions in fatally infected animals were altogether typical and hence will not be described in detail except in unusual instances. Leptospiras were found in the organ emulsions and a culture was obtained from the blood.

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<sup>5</sup> Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 253.

Transfer<sup>v</sup> from one of these animals was made into three guinea pigs, all of which succumbed in due time to typical fatal infection.

One of the guinea pigs (No. 25) was used, when near collapse, for testing the efficacy of the anti-*icteroides* serum. The animal recovered, having received 1 cc. of the serum.

*Case 12 (Fatal).*—P. C., male, age 28 years; native of mountainous region. Onset, Apr. 23, 1920, typical. Apr. 25. Seen for the first time; temperature 39.4°C.; pulse 102; albumin ++; epigastric pain; no vomit. Apr. 26. Temperature 34.8°; patient in state of collapse; bleeding from nose and gums; black vomit; jaundice. Apr. 27, 6 a.m. Died.

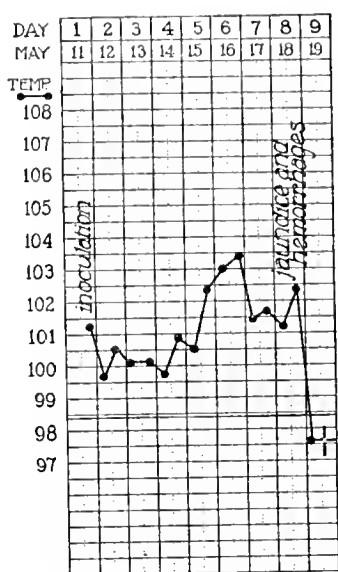
Blood was taken in the morning of the 3rd day of illness. Cultures contained living leptospiras when examined on May 7 (12 days old).

May 6. Six guinea pigs (Nos. 1 to 6) were inoculated with culture material. Of these, three (Nos. 2, 3, and 4) developed severe infections, one dying on the 7th, and one on the 8th day (Chart 3), and the third being killed for transfer on the 6th day, when it was intensely icteric. Three guinea pigs inoculated with blood and liver and kidney emulsions from this animal died with typical symptoms. Three of the six original guinea pigs showed no sign of infection (Nos. 1, 5, and 6), but examination after 12 days revealed hemorrhagic areas in the lungs and also, in one instance, in the suprarenal. Leptospiras were found in the blood and organs in some of the guinea pigs, and cultures were obtained from the blood. The outstanding feature of this strain was the predominance of jaundice which it produced in the animals.

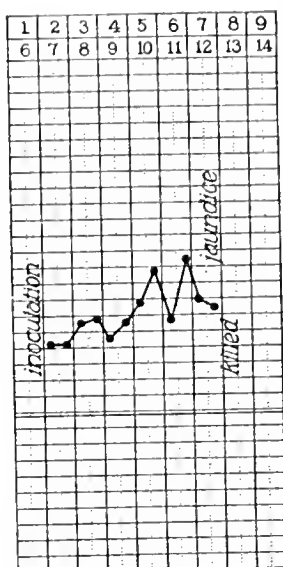
*Case 13 (Moderate; Recovered).*—J. C., male, age 14 years; native of Morropon. Onset Apr. 23, 1920. Apr. 26. Seen for the first time; epistaxis; black vomit; melena; temperature 37°C.; pulse 80; albumin ++. Apr. 27. Temperature 36.6°; pulse 66; albumin ++. Apr. 28. Temperature 37°; pulse 80; albumin ++. Recovery.

Apr. 26 (4th day). Blood taken for cultures. Dark-field examination of cultures 11 days after they were made failed to reveal any leptospira. Six guinea pigs inoculated with the 10 day culture material from this case also yielded negative results.

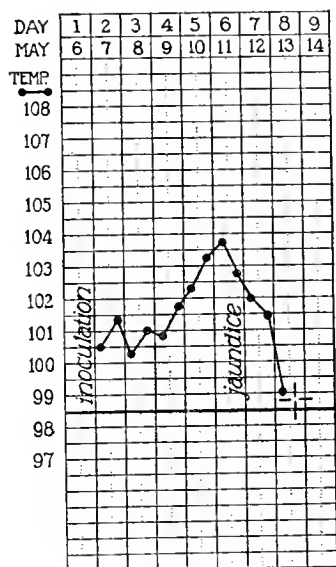
*Case 14 (Severe; Recovered).*—F. N., female, age 25 years; native of Morropon. Apr. 25, 1920, 11 a.m. Onset. Apr. 26. Temperature 38.5°C.; pulse 114; albumin trace; headache; backache; muscular pain; face flushed; conjunctivæ congested. Apr. 27. Temperature in morning 39.2°; pulse 106; albumin +. 4 p.m. Temperature 39.5°; pulse 120; severe pain and weakness; nausea, but no vomiting. At request of patient 20 cc. of anti-*icteroides* serum brought from The Rockefeller Institute were injected intravenously. 7 p.m. Temperature 38.9°; pulse 100; stronger; pains relieved. Apr. 28. Afternoon temperature 38.2°; pulse 100; albumin ++; nausea; bilious vomit. Apr. 29. Temperature 38.2°; pulse 90; no nausea; albumin ++. Apr. 30. Temperature 37.9°; pulse 86; mild pharyngitis. May 1. Temperature 36.8°; pulse 80; albumin +; recovering.



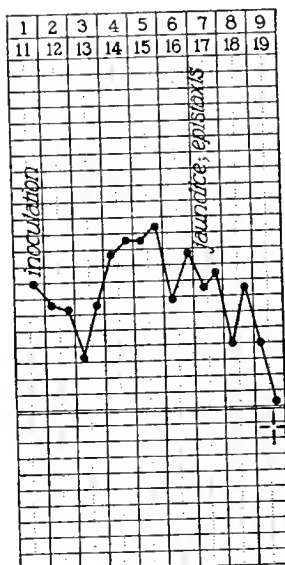
GP 39 (Strain 1-Case 10).  
Chart 1.



G.P. 26 (Strain 2-Case 11).  
Chart 2.



GP. 2 (Strain 3-Case 12).  
Chart 3.



GP. 45 (Strain 4-Case 14).  
Chart 4.

CHARTS 1 TO 4. Temperature curves of guinea pigs inoculated with material from yellow fever cases in Peru.

Apr. 27 (48 hours after onset). Blood taken in the morning. The cultures were examined on May 8 when 11 days old, and living leptospiras were found in one tube, apparently dead ones in another.

May 6. Six guinea pigs (Nos. 7 to 12) were inoculated with the 9 day culture material. A typical infection was induced in three, while the other three remained apparently well. One of the latter showed hemorrhagic areas in the lungs when examined after 12 days. The other two had no macroscopic lesions, indicating that they were both completely refractory to this strain.

Transfer was made from one of the positive animals into three guinea pigs, all of which died with typical infections. Leptospiras were demonstrated in varying number in the blood as well as in the emulsions of liver and kidneys, and pure cultures were obtained from the blood of these animals. Chart 4 shows the temperature curve of one of the guinea pigs (No. 45) infected with Strain 4.

In Case 14, as in Cases 10, 11, and 12, positive transmission to guinea pigs was obtained by means of culture material. The initial cultures usually contained living leptospiras. In the blood and liver or kidneys of the infected animals the leptospiras were demonstrated, and a pure culture of *Leptospira icteroides* was recovered from the blood.

#### *Identification of the Morropon Strains.*

Upon our return to The Rockefeller Institute, we proceeded with the identification of the strains of leptospira isolated from yellow fever cases in Morropon along the lines previously followed. The Pfeiffer phenomenon was determined, as well as the effects of immune serums upon the organism *in vitro*.

Rich cultures of leptospira strains (Nos. 1 and 2) were employed in these experiments. The serums used were monovalent immune serums prepared in rabbits with Guayaquil Strain 1 of *Leptospira icteroides*. For purposes of control American Strain 2 of *Leptospira icterohæmorrhagiæ* was tested simultaneously. Moreover, a polyvalent immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides* was also tested. The results obtained are given in Table I.

As Table I shows, the leptospira strains from Morropon gave positive Pfeiffer reactions with the immune serums prepared with the Guayaquil strains, but negative reactions with the anti-*icterohæmorrhagiæ* serum. Likewise, with respect to their behavior towards

these serums *in vitro* an indubitable specificity for the anti-*icteroides* serum is evident. The slight reaction with the anti-*icterohæmorrhagiæ* serum may be regarded as a group reaction among closely allied species. It is concluded, therefore, that the leptospiras isolated from Morropon and Guayaquil cases of yellow fever are of the same species.

TABLE I.  
*Identification of the Morropon Strains.*

Mode of test.	Strain No.	Anti- <i>icteroides</i> serum (monovalent, rabbit).	Anti- <i>icteroides</i> serum (polyvalent, horse).	Anti- <i>icterohæmorrhagiæ</i> serum (monovalent, rabbit).	Controls without immune serum.
Pfeiffer (30 min.).	1	Complete disin- tegration (positive).	Complete disin- tegration (positive).	For the most part active (negative).	Very active (negative).
	2	Almost com- plete disin- tegration (posi- tive).	Complete disin- tegration (positive).	For the most part active; few appear distorted (negative).	Very active (negative).
<i>In vitro</i> ; al- lowed to stand for 18 hrs.	1	Complete ag- glutination; some motile leptospiras.	Complete ag- glutination and immobil- ization.	Slight aggluti- nation and few immobil- ized.	Very active; no agglutination.
	2	Complete ag- glutination; some motile leptospiras.	Complete ag- glutination; few motile leptospiras.	Partial aggluti- nation and immobiliza- tion.	Active; no agglu- tination.

#### DISCUSSION AND SUMMARY.

Fourteen typical cases of yellow fever were studied in northern Peru during an epidemic occurring in 1920, nine in Payta in March and April, and five in Morropon and Piura in April and May. The method of investigation was similar to that previously employed, but as the laboratory facilities were very meager certain changes were required. Although in Payta the work was handicapped by the lack of electric light, the scarcity of water and animal food, the unsuitability of the guinea pigs for inoculation, and the changes in culture media due to age, the results obtained under these adverse conditions were by no means negative. While in no instance was there a typical



infection produced in animals, either by direct inoculation of blood or with culture materials, yet certain guinea pigs in each series showed temporary febrile reactions or definite hemorrhagic lesions of the lungs indicative of a mild leptospira infection. Direct search for *Leptospira icteroides* in the blood of patients or in culture materials was not made because the dark-field microscope could not be used.

Subsequently, at Piura, the laboratory facilities were vastly improved, the use of the dark-field microscope was made possible by means of a storage battery, and a fresh stock of young healthy guinea pigs was received from New York, and fresh rabbit serum obtained in Piura. In the study of the materials obtained from five cases of yellow fever in Morropon all these added facilities were taken advantage of, with the result that the outcome was positive and convincing. Cultures from the five cases were examined after 11, 12, and 13 days, and in those from three cases living leptospiras were found.

By inoculation into suitable guinea pigs of culture material from these five cases, irrespective of whether or not leptospiras were detected under the dark-field microscope, a typical *Leptospira icteroides* infection was produced from four of the five cases. In one of these no leptospira had been detected in the culture tubes. Thus one case only yielded negative results, in that no leptospiras were found under the dark-field microscope and the animal inoculation was negative.

The leptospira was demonstrated in the blood or organ emulsions of the infected guinea pigs, and further transmission of each strain to other guinea pigs was obtained and pure cultures were secured.

A few points of practical significance appeared in the course of the present investigation. One is the importance of using fresh rabbit serum for culture media. Old rabbit serum, whether in pure form or incorporated with agar, etc., which had been kept for several months in a tropical climate, proved to be unsatisfactory for obtaining a growth of *Leptospira icteroides*. A second point of interest is the variation in susceptibility of guinea pigs to infection with *Leptospira icteroides*. In two of four series of positive animal inoculations with the Morropon culture materials only one-half of the guinea pigs inoculated with given materials developed typical symptoms. The other half either suffered from a transient mild infection, as evidenced by a few hemorrhagic foci in the lungs, or escaped infection altogether.

From these facts it is highly probable that the lung lesions and febrile reactions observed in certain guinea pigs inoculated with the Payta materials were due to a mild leptospira infection. In a comparative experiment the native guinea pigs procured in Payta were found to be more resistant to the leptospira infection than those recently brought from New York. In fact, only a small portion of the former succumbed to typical infection even when inoculated with a virulent strain of *Leptospira icteroides* obtained from the Morropon epidemic.

In conclusion it may be stated that of fourteen cases of yellow fever studied in Peru, a typical leptospira infection, together with the demonstration of the organism in experimentally infected guinea pigs, was obtained in four, while in the majority of instances indications of a mild, non-fatal leptospira infection were observed. In a few cases only were the results entirely negative.

The leptospira isolated from Morropon cases of yellow fever, which is morphologically and culturally identical with the Guayaquil and Merida strains of *Leptospira icteroides*, was also shown by immunity test to be indistinguishable from the Guayaquil organism.

## IMMUNOLOGY OF THE PERUVIAN STRAINS OF LEPTOSPIRA ICTEROIDES.

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(Received for publication, August 7, 1920.)

In the course of a study of the etiology of yellow fever in Peru,<sup>1</sup> an effort was made to solve several related problems of the disease. The first point taken up was the behavior of the serums of Peruvian convalescents in Pfeiffer's reaction towards strains of *Leptospira icteroides* isolated elsewhere; the second concerned the action, *in vitro* and *in vivo*, on the Peruvian strains of the leptospira, of a polyvalent immune serum prepared in horses with several strains of *Leptospira icteroides* of Guayaquil origin; the third related to the difference in the natural resistance to the leptospira infection of the native guinea pigs as compared with guinea pigs recently brought from New York; and the fourth and last point bore on the availability of old rabbit serum for cultivating *Leptospira icteroides*. The last two questions had arisen as a result of the rather unfavorable outcome of the work carried out in Payta, and, as will appear later, proved to be important.

### *Pfeiffer Reaction with Serum from Convalescents.*

The method was similar to that previously employed. 1 cc. of each serum was mixed with 0.2 cc. of a rich 6 week *icteroides* culture of Guayaquil Strain 1 and the Merida strain. The guinea pigs used were those recently brought from New York. Observations were made at the end of 1 hour.<sup>2</sup>

<sup>1</sup>Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 239.

<sup>2</sup>This work was done at the Belan Hospital, Piura, where we received cordial cooperation from Dr. M. Guzman R., Dr. A. Gonzalez, and Dr. Prieto, as well as the mother superior. We also wish to thank especially Dr. Marcos L. Vega, the government sanitary officer at Piura, for the arrangement to test the convalescents in Piura.

Nine convalescents were accessible for this test. Of these, four came from the Payta (1920), four from the Piura (1919), and one from the Morropon (1920) epidemic. The serums were obtained in the five 1920 cases within a period ranging from 7 to 36 days from the time of onset of the fever and in the four 1919 cases at the end of 10 to 11 months. The results obtained are shown in Table I. The interpretation of the findings is evident. All the Peruvian cases, except two instances to be discussed below, gave a positive Pfeiffer reaction to two different strains of *Leptospira icteroides*, irrespective of whether the case occurred in Payta, Piura, or Morropon.

TABLE I.  
*Pfeiffer Reaction with Serum from Convalescents.*

Serum No.	Locality of epidemic.	Length of time between onset of yellow fever and withdrawal of blood.	Strain of <i>Leptospira icteroides</i> .	Results.
		<i>days</i>		
1	Payta.	7	Guayaquil No. 1	Positive.
2	"	7	" " 1	"
3	"	36	" " 1	"
4	"	17	" " 1	"
		<i>mos.</i>		
5	Piura.	11	Merida.	"
6	"	10	"	"
7	"	10	"	Doubtful.
8	"	10	"	Partial reaction.
		<i>days</i>		
9	Morropon.	21	"	Positive.
Control.			"	Negative.

The exceptions were the serums obtained from two persons who had had yellow fever 10 months previous to the time of testing, both giving a slight or partial reaction. It may be noted, however, that two other individuals who had had an attack 10 and 11 months previously reacted positively. The reason for a partial reaction in the first two cases probably relates to a gradual diminution of the antibodies responsible for the Pfeiffer reaction.

The foregoing observations are of value in establishing the identity of the organisms that were present in individuals suffering from yellow fever in Ecuador, Mexico, and Peru. The positive Pfeiffer

reaction with the serums of yellow fever convalescents in Payta also indicates that the guinea pigs of the Payta experiments which had shown suspicious febrile reactions and hemorrhagic lesions of the lungs and kidneys without developing a typical fatal infection, had actually suffered a mild infection with *Leptospira icteroides*. Finally, it may be added that the guinea pigs which were employed in the Pfeiffer tests and reacted positively showed no symptoms and survived, while the control animal (inoculated with the Merida strain) suffered from a typical but non-fatal infection.

### Virulence.

The virulence of the Morropon strains was next tested on New York guinea pigs. The material used was an emulsion of kidney

TABLE II.

*Determination of the Minimum Lethal Dose of Morropon Strains.*

Amount of kidney emulsion in 1 cc.	Strain 1.		Strain 3.	
	Incubation period.	Result of inoculation.	Incubation period.	Result of inoculation.
cc.	days		days	
1	4	Died in 9 days.	No test made.	No test made.
0.1	4	" " 8 "	3	Died in 8 days.
0.01	3½	" " 7 "	4	" " 9 "
0.001	5	Recovered.	3	" " 7 "
0.0001	6½	Died in 11 days.	5	" " 8 "
0.00001		No infection.	5	" " 10 "

The animals used for this experiment served at the same time as controls for the therapeutic experiments reported in Table III.

made in saline solution in a ratio of approximately 1 gm. to 10 cc. After the coarse tissue fragments had settled, the turbid supernatant portion was employed for inoculation. Ascending tenfold dilutions were prepared with 0.9 per cent saline solution, and 1 cc. was injected intraperitoneally into guinea pigs, one animal being used for each dilution. Table II gives the minimum lethal dose as determined for Morropon Strains 1 and 3. The approximate minimum lethal dose for Strain 1 lies between 0.0001 and 0.00001 cc., and that of Strain 3 beyond 0.00001 cc. The grade of virulence of the strains is, therefore, about the same as that of strains previously studied.

*Therapeutic Experiments with Anti-icteroides Serum.*

The reaction of the Peruvian strains towards the anti-*icteroides* immune serum prepared with the Guayaquil strains confirms the other evidence of the identity of the organisms. It has been shown in our first paper<sup>1</sup> that the leptospira from Peru gave a positive Pfeiffer reaction with this immune serum. In the present series of experiments the object was to determine, in guinea pigs given multiple minimum lethal doses of Morropon Strains 1 and 3, the prophylactic and curative values of a specific polyvalent anti-*icteroides* immune horse serum. The immune serum was administered in varying quantities and at different periods after inoculation, even after the appearance of symptoms, up to the time when the animals were fast approaching the terminal stage of the disease induced. The guinea pig kidney emulsions used for infecting the animals were injected intraperitoneally in uniform amounts of 0.2 cc., this dose representing at least 2,000 minimum lethal doses of Strain 1 and 20,000 minimum lethal doses of Strain 3. The immune serum was given intraperitoneally also in doses of 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc., contained in saline solution to a total volume of 1 cc., and finally 1 cc. of undiluted serum. In order to obviate the factor introduced by an occasional unusually refractory animal, more than two of the recently brought guinea pigs were used in testing the effect of each dose of serum. Table III, which summarizes the results, brings out the following points. (a) The anti-*icteroides* serum is capable of checking the development of the infection, provided a sufficiently large quantity is given during the incubation period. The four deaths which took place among animals treated during this period occurred among those which did not receive serum until 72 hours after inoculation; *i.e.*, at the end of the incubation period. (b) A quantity of serum larger than 0.01 cc., given during the febrile stage (96 hours after inoculation) usually prevented the infection from reaching the icteric stage. This was true particularly with the animals infected with Strain 1, which had scarcely a tenth of the virulence of Strain 3. (c) The effect of the serum became uncertain when it was given during the icteric stage (5 to 6 days after inoculation). With 0.01 cc. no animal was saved, while 0.1 cc. saved three out of

four infected with Strain 3, but none with Strain 1. 1 cc. of undiluted serum saved one out of three with Strain 1 and three out of four with Strain 3. That the proportion of recoveries among the animals treated during the icteric stage does not correspond with the virulence of the strains can be explained only on the basis of the natural resistance of individual guinea pigs; at all events, death occurred in both groups, even when 1 cc. of serum was given.

TABLE III.

*Protective and Curative Effect of Anti-icteroides Serum in Guinea Pigs Experimentally Infected with Leptospira icteroides.*

Strain.	Amount of serum.	Animals treated before beginning of fever.			Animals treated during febrile stage.			Animals treated after jaundice appeared.		
		Total.	Remained well.	Died.	Total.	Recovered without jaundice.	Died.	Total.	Recovered.	Died.
0.2 cc. of Strain 1 culture, representing 2,000 minimum lethal doses.	cc.									
	0.0001	2	1	1						
	0.001	5	5	0	3	0	3			
	0.01	4	4	0	5	5	0	2	0	2
	0.1	4	4	0	3	3	0	2	0	2
	1				4	4	0	3	1	2
0.2 cc. of Strain 3 culture, representing 20,000 minimum lethal doses.	0.0001	2	2	0						
	0.001	6	5	1	2	0	2			
	0.01	6	4	2	5	3	2	2	0	2
	0.1	3	3	0	3	3	0	4	3	1
	1				2	2	0	4	3	1

The controls without serum treatment are represented by the animals of the experiment recorded in Table II.

*Resistance of Native and Imported Guinea Pigs to Leptospira icteroides.*

Certain tests made with guinea pigs obtained in Payta suggested that the native Peruvian guinea pigs are more refractory to the leptospira than guinea pigs brought from New York. To determine this point four groups of guinea pigs were inoculated, on May 16, 1920, with a uniform amount of kidney emulsion from a guinea pig which had shown a typical infection with Morropon Strain 4. The

animals inoculated comprised: (1) fifteen native guinea pigs which had once been inoculated with culture materials in Payta, without any definite infection having been induced; (2) six native guinea pigs which had not been used; (3) eight American guinea pigs which had formerly been inoculated with culture materials in Payta, without infection having been induced; and (4) four newly imported American guinea pigs. Table IV summarizes the result. It will be noted that the virulence and dose of the strain used were such that even the young American guinea pigs, used as a standard of susceptibility, did not all develop a fatal infection. This effect was rather an advantage, since, had multiple fatal doses been given, the difference in susceptibility might not have come out so clearly.

TABLE IV.

*Relative Resistance of Native and American Guinea Pigs to Leptospira icteroides.*

Guinea pigs.	Total No. injected.	Escaped infection (no jaundice).	Animals showing definite infection.*	
			Recovered.	Died.
Native (once used).....	15	9	1	5
“ (normal).....	6	6		
American (old lot; once used).....	8	3	3	2
“ (new lot; normal).....	4	0	2	2

\* This includes guinea pigs which showed typical lesions when killed.

The striking feature of the foregoing experiment is the complete resistance of all six normal native guinea pigs in contrast to the four normal American animals, of which two, at least, died of a typical infection. Among the animals inoculated on a former occasion, both the native and the American groups contained susceptible as well as refractory ones, the difference being that a larger proportion of the native (60 per cent) than of the American (37.5 per cent) proved resistant.

The relatively large proportion of resistant animals among the native guinea pigs, as well as among the American guinea pigs which had withstood the unfavorable climate and other hardships, is a point to be taken into account in future work.



*Deterioration of Rabbit Serum through Age.*

That an alteration takes place in rabbit serum when it is subjected to long transportation in a hot climate is evident from the fact that no growth of *Leptospira icteroides* could be obtained with such serum. On the other hand, old rabbit serum which has been kept in the refrigerator up to 6 weeks has been found still suitable for making subcultures of *icteroides*. Whether the same would be true after 2 or 3 months standing at 4°C. has not, however, been determined.

The rabbit serum which was used in the cultivation experiments in Payta<sup>1</sup> was 3 months old and had been exposed to the ordinary tropical temperature of the region, which is sometimes 40°C. during the day, and it contained considerable precipitate. It was thought probable that the deterioration of the serum through age and climatic conditions might have been a factor in the unsuccessful outcome of the Payta experiments. Moreover, another lot of serum, which had been collected in New York in April and hermetically sealed, was brought down to Piura under ordinary temperature conditions. Some tubes contained precipitate, while others had remained clear. It was used when 50 days old for making subcultures of four Morropon strains of the leptospira, but when the culture tubes were examined 10 days later no growth could be found. Fearing a possible loss of all strains, we made two more sets of subcultures of the same strains, one set with the old and another with fresh rabbit serum. With the media containing the fresh rabbit serum a rich growth took place with three of the four strains, but there was no growth in the tubes prepared with the old serum.

Other factors, however, made it difficult to determine how important it is in general to employ fresh serum in culture work with *icteroides*. The Peruvian strains were unusual in that they early degenerated in culture. The initial cultures from human cases never became very rich, and they disappeared within about 3 weeks. The first generation cultures from the infected guinea pigs grew fairly well within a week, but at the end of 2 weeks examination showed only a few active organisms and many degenerating. Of the cultures of Strain 3, none was alive, and injection into guinea pigs failed to recover it. The remaining strains (Nos. 1, 2, and 4) were successfully

subcultured by using fresh rabbit serum, as noted above. It is certain that all the strains would have been lost if we had relied entirely on the old serum. The present study does not indicate whether the importance of using fresh serum applies only to the primary human and recently isolated generations or even to remote subcultures.

#### SUMMARY.

Serum from yellow fever convalescents from Payta, Piura, and Morropon gave a positive Pfeiffer reaction with the strains of *Leptospira icteroides* isolated in Guayaquil and Merida. The serum also protected the guinea pigs from these strains in the majority of instances. The Pfeiffer reaction was complete with all recent convalescents (7 to 36 days) but slight or partial in some instances with serum derived from individuals who had had the attack of yellow fever 10 months previously.

The virulence of the Morropon strains was found to be approximately the same as that of the Guayaquil or Merida strains. With one strain the minimum lethal dose for the guinea pig was less than 0.00001 cc. of a kidney emulsion from an infected guinea pig.

Suitable quantities of the anti-*icteroides* serum administered to guinea pigs inoculated with 2,000 to 20,000 minimum lethal doses of infective material prevented the development of the infection, or a fatal outcome, according as the serum was given during the incubation period or after fever had appeared. The earlier the administration of the serum the smaller was the quantity needed; during the incubation period 0.0001 to 0.001 cc. was sufficient, during the febrile period 0.01 to 0.1 cc. was required to check the progress of the disease, and even at the time when jaundice had already appeared, the injection of 0.1 to 1 cc. saved three out of four animals inoculated with Strain 3 and one out of three inoculated with Strain 1.

The native guinea pigs secured in Payta proved to be unusually refractory to infection with *Leptospira icteroides* as compared with normal guinea pigs recently imported from New York.

Fresh rabbit serum is recommended for culture work with *Leptospira icteroides*.

## MIGRATION OF LYMPHOCYTES IN PLASMA CULTURES OF HUMAN LYMPH NODES.

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PLATES 10 AND 11.

(Received for publication, October 7, 1920.)

In this study plasma cultures were made from normal and pathological lymph nodes obtained from operations at the Johns Hopkins Hospital. The method was as follows: Pieces were dropped into Locke-Lewis solution, and while in the solution were cut into small fragments 0.5 mm. or less in diameter. About 5 cc. of blood, withdrawn into a small paraffin-lined test-tube, were kept cool in a freezing mixture before centrifugalization. The centrifugized blood was kept in the original tube surrounded by the freezing mixture and the plasma drawn off as the cultures were made. Cultures were made as rapidly as possible, in the usual manner, the number varying from 20 to 60 in the different series. Sometimes, however, 2 hours elapsed from the time the node was removed until the final cultures were made. The differences in time did not seem to affect the outgrowth from the explant. Both auto- and homoplasma were used, but no differences in the outgrowths were observed.

The lymphocytes were almost always the first cells to migrate out into the plasma clot. In autoplasma migration was observed within 1 to 2 hours, and in homoplasma  $1\frac{1}{2}$  to  $2\frac{1}{2}$  hours after the cultures were put in the warm box. The number of series of cultures was not sufficient to determine whether the initial latent period varies with the type of plasma and of tissue. The lymphocytes continue to migrate out into the clot for 2 or more days, or until practically all those which are living have left the explant. They migrate out into the plasma clot much more readily than onto the cover-slip over the liquefied areas. Liquefaction of the clot, as a rule, was delayed for

a day or two and varied greatly in extent. In some of the cultures however, the process began almost immediately after the cultures were made and in the course of an hour the explants were more or less surrounded by fluid. In these cultures lymphocytes were seen migrating out into the part of the clot which still remained attached to the explant. Comparatively few lymphocytes, on the other hand, migrated out onto the cover-slip over the liquid area. In some instances one portion of the clot was almost free from lymphocytes while another portion, attached to the explant, was full of them. Lymphocytes which came to the surface of the explant bordering the liquefied area fell to the bottom of this area, as many spherical lymphocytes were seen on the lower surface of the liquid.

The lymphocytes which appeared on the cover-slip over the liquefied area were generally associated with the large wandering cells (Figs. 6, 8, and 9) and with the giant cells. As the last two types of cells migrated out from the explant they occasionally carried with them one or more lymphocytes, the number sometimes being very large, which moved about on the under, free surface of the large cells and tended to remain there, rather than to wander off onto the cover-slip. Small lymphocytes could often be seen creeping around on a large giant cell, or moving back and forth in various directions, reaching the edge of the cell but rarely leaving it to proceed onto the cover-slip; they sometimes came to rest, assuming a rounded form, but still remained attached to the large cell. Apparently they adhered more readily to the flat wandering cells and giant cells than to the cover-slip. In fixed specimens the lymphocytes remained attached to these large cells (Figs. 8 and 9).

Lymphocytes sometimes tended to clump together in clusters of half a dozen or more. This clumping usually took place in the neighborhood of either small wandering cells or some foreign body. It resembled an agglutination without actual fusion of the cells. Only comparatively few of the lymphocytes tended to adhere together in this manner. The same phenomenon was observed in cultures (1) from normal nodes in both autoplasm and homoplasm, (2) from lymphadenitis specimens in autoplasm, (3) from tuberculous nodes in homoplasm, and (4) from metastatic sarcomatous nodes in homoplasm.

*Paths of Migration.*

In general, the lymphocytes migrated away from the explant and tended to spread out radially from it. When the route pursued by individual lymphocytes was observed in detail, however, it was found that they did not progress in direct radial lines away from the explant but moved in very irregular paths, as shown in Figs. 1 to 5. They sometimes made complete circles within a diameter of 0.033 mm. or less, or came almost to rest and turned and twisted about in a space of 0.006 mm. or less. They often migrated more or less rapidly for a few minutes, then came to rest, assumed a rounded form, and remained stationary for varying periods of time, from a few minutes to an hour or more. When the lymphocytes began to migrate again they did not always continue in the same direction. They crossed one another's paths without apparent deflection, or came into contact with one another without adhering. Contact with other cells or fibrous threads caused more or less deflection from the original course. It was not uncommon to see a lymphocyte come squarely against a strong fibrous thread with force enough to indent deeply the nucleus so as almost to divide it in two, remain there for a few seconds, and then slide off to one side or the other and continue its general course, the nucleus returning to its original form. The nuclei seemed semi-solid and very plastic, and continually changed shape as the cells met one obstruction after another; even when no obstruction was encountered, the nuclei underwent many changes in form as the cells twisted, turned, and elongated during migration. Sometimes the lymphocytes came up towards the cover, or penetrated into the depths of the clot, or migrated between the clot and the cover-slip; but as stated before, they generally migrated in the clot away from the explant. They did not appear to follow the fibrin threads but occupied the spaces between the latter. Migration from the explant continued until very few or only dead ones were left. There were always, of course, many more per cubic millimeter near the explant than towards the periphery of the clot, with occasional exceptions in old cultures.

TABLE I.

Series.	Distance traversed in successive min.	Rate of travel per min.		
		Maximum.	Minimum.	Average.
		mm.	mm.	mm.
(17) Carcinoma in homoplasma. 1½ hr. culture; 39°C. (Fig. 4).	(1) 8-4-13-9-8-2-5*	0.0173	0.0026	0.0093
(15) Normal node in homoplasma. 4 hr. culture; 38°C.	(2) 4-11-15-11	0.02	0.0053	0.0136
	(3) 11-13-10	0.0173	0.0133	0.0151
	(4) 9-15-13-4-7-9	0.02	0.0053	0.0113
(8) Metastatic sarcoma in homoplasma. 4 hr. culture.	(5) 18-12-12-18-12-17	0.0243	0.016	0.02
(12) Lymphadenitis specimen in homoplasma. 24 hr. culture; 38.5°C. (Fig. 3.)	(6) 4-15-6-6-7-12-9-8-9-12-17-12-8-9-5-14-21-13	0.028	0.0053	0.0133
	(7) 4-8-10-7-14-8-9-14-12-9-8	0.0186	0.0053	0.0126
	(8) 0-2-3-12-7-6-8-10-21-9	0.028	0.0026	0.0111
	(9) 8-12-8-5-14-9-6-10-13-9-9-8	0.0186	0.0066	0.0125
	(10) 5-7-6-10-5-6-2-9-9-7-11	0.0146	0.0026	0.0093
	(11) 18-15-12-?-?-?-11-15-12-22-7-8	0.0293	0.0093	0.0178
	(12) 8-5-17-5	0.0227	0.0066	0.0111
(16) Normal node in homoplasma. 24 hr. culture; 39-40°C.	(13) 8-7-10-9-8-8-8	0.0133	0.0093	0.0109
	(14) 8-5-9-8-7-6-8-7	0.0120	0.0066	0.0152
	(15) 4-10-5-8-10-13-6-5-8-11	0.0173	0.0053	0.0106
	(16) 17-11-12-6-15-18	0.024	0.008	0.0173
	(17) 7-12-3-8-4-10-3-5-11-8-5-6-15-12-11-8-5-9	0.02	0.004	0.0106
(13) Normal node in homoplasma. 20 hr. culture; 37.5°C. (Fig. 2).	(18) 6-9-6-5-4-7-5-0-0-0-8-6-3-6-7-7-10-3-6-6-2-7-2-8-5-0-0-0-2-2-2-1-8-6-7-0-0-0-5-5-0-0-0-0-0-2-4-4-5-6-0-0-0-0-0-0-0-	0.0133	0.0013	0.0069
(18) Tuberculous node in homoplasma. 48 hr. culture.	(19) 8-21-16-10-15-14-8-12	0.028	0.0106	0.0173

\* The figures for the distance traversed are given in millimeters multiplied by 750.

TABLE I—*Concluded.*

Series.	Distance traversed in successive min.	Rate of travel per min.		
		Maximum.	Minimum.	Average.
		mm.	mm.	mm.
(8) Metastatic sarcoma in homoplasma. 48 hr. culture; 38.5–39.5°C.	(20) 8–14–12–12–?–?–0–0–0–0 for 30 min.	0.0186	0.0106	0.0152
	(21) 10–12	0.016	0.0133	0.0146
	(22) 9–8–8–7–10–7	0.0133	0.0093	0.0113
	(23) 18 (Fig. 1)	0.025		
	(24) 12–10–10–6	0.016	0.008	0.0126
	(25) 10–9–6–3	0.0133	0.004	0.009
(12) Lymphadenitis specimen in homoplasma. 44 hr. culture.	(26) 7–15–15–14–11	0.02	0.0093	0.0165
	(27) 13–1–5–11	0.0173	0.0013	0.01
(9) Tuberculous node in autoplasm. 4 day culture (Fig. 5).	(28) 6–6–5–5	0.008	0.0066	0.007
	(29) 5–5–5–4–2–3–5–7	0.0093	0.0026	0.006
	(30) 5–8–2–9–8–11–6	0.0146	0.0026	0.0093
	(31) 5–5–2–6–6–10	0.0133	0.0026	0.0096
(18) Tuberculous node in homoplasma. 7 day culture; 37.5°C.	(32) 10–12–9–6–5–8–3–4–3–8	0.016	0.004	0.0077
	(33) 7–5–6–8–11–11–10–12	0.016	0.0066	0.0116
	(34) 10–13–12	0.0173	0.0133	0.0154
	(35) 5–4	0.0066	0.0053	0.006

*Rate of Migration.*

The rate of migration was studied by camera lucida drawings made at minute intervals with a magnification of 750 or 1,250 diameters. Most of the lymphocytes migrated out of the field of the camera in a few minutes, so that the period of observation for any one lymphocyte was usually rather short. There was great irregularity in the distance covered during successive intervals. The greatest distance traversed during 1 minute was 0.03 mm., the maximum distances for the different lymphocytes varying from, 0.03 to 0.006 mm. The minimum varied much more; namely, from 0.013 to 0.0013 mm. per minute. This was to be expected, since lymphocytes often slowed down before they came to rest. The average rate varied from 0.02 to 0.006 mm. per minute. Determinations were made in cul-

tures from 1½ hours to 7 days old, but no especial differences were noted in the rate of migration. Measurements were also made of lymphocytes (1) from normal nodes in homoplasma, (2) from lymphadenitis specimens in homoplasma, (3) from tuberculous nodes in auto- and homoplasma, (4) from metastatic sarcomatous nodes in homoplasma, and (5) from a metastatic carcinomatous node in homoplasma, but the rate of migration was essentially the same in all, as shown in Table I. The observations were not extensive enough to draw conclusions as to the meaning of the variations in rate in cultures of different ages or in the different combinations of plasma and explant. We have not been able to discover any differences in lymphocytes from normal and pathological nodes.

Hirschfeld<sup>1</sup> in 1901 observed the ameboid activity of lymphocytes from a case of lymphatic leucemia, using Deetjen's method by placing a drop of leucemic blood on a film of 1 per cent agar which contained 0.6 gm. of sodium chloride, 6 to 8 cc. of a 10 per cent solution of sodium metaphosphate, and 5 cc. of a 10 per cent solution of dipotassium phosphate, per 100 cc.

### *Structure.*

The appearance of the cytoplasm of the living lymphocyte differed from that of the other cells. This was more noticeable in the elongated moving lymphocyte. It is difficult to describe other than that it was darker and less transparent. The cytoplasm seemed to be rather firm in consistency, since it rarely spread out into a very thin layer like that of the wandering cells. The lymphocytes in fresh cultures contained very few granules, and with neutral red only two or three granules at most became red. In older cultures there was a gradual increase in the number of granules which took up the neutral red. They were always small and did not seem to have vacuoles about them. The mitochondria were difficult to see unless colored with Janus green or Janus black No. 2. They were few in number, granular in form, and small. Lymphocytes often contained one or more fat globules (Fig. 1). In the migrating lymphocytes one could usually distinguish a homogeneous ectoplasm and a darker endoplasm which contained the granules and mitochondria.

<sup>1</sup> Hirschfeld, H., *Berl. klin. Woch.*, 1901, xxxviii, 1019.



The nucleus had a peculiar waxy appearance; it appeared to be homogeneous or structureless, and the nucleolus was very difficult to discern.

### *Shape.*

The resting lymphocytes (Figs. 1, 6, and 7), and those that had fallen to the bottom of the liquefied area, were usually more or less spherical and presented the characteristic picture of a thin shell of cytoplasm enclosing a comparatively large nucleus eccentrically placed. The migrating or moving lymphocytes, on the other hand, had a peculiar, elongated shape, with the nucleus always at or very near the anterior or forward-moving end (Figs. 1 to 5 and 8 to 10). The cells seemed to possess a distinct polarity. The degree of elongation varied during successive minutes, but the nucleus was always near the anterior end with a small amount of ameboid cytoplasm in front which was continually changing shape and was sometimes so small in extent as to be scarcely visible. This anterior cytoplasm was homogeneous and free from granules. There was usually very little cytoplasm at the side of the nucleus; the bulk of it, in these migrating cells, lay behind the nucleus and was more or less finger-shaped, consisting of a thin ectoplasm enclosing an endoplasm which contained mitochondria and granules. The nuclear region was often the broadest part of the cell, but the nucleus itself was very plastic and was continually changing shape, although it seemed to be more solid than the cytoplasm.

When the lymphocytes came to rest and assumed a rounded form, the large cytoplasmic tail formed the thicker part of the cytoplasmic rim and forced the nucleus into a slightly eccentric position; when the lymphocytes began to move and stretched out, this thickened part of the cytoplasmic rim formed the elongated tail. The resting as well as the migrating cells possessed a distinct polarity.

We were not able to distinguish a centriole or a central area as in the mesenchyme cells of chick embryos, so that it was impossible to determine whether polarity had any relation to a centriole. The living lymphocytes in the plasma clot were remarkably constant in size, shape, and structure, and showed no tendency toward transition to other types of cells. They were easily recognized, either at rest

or during migration, and their general appearance was so distinctive that they were never confused with the other types. Occasionally a large mononuclear cell was seen which resembled a lymphocyte in every particular except size. The general appearance of the cytoplasm and nucleus was similar. They migrated with the nuclear end first and were then elongated, but assumed a more or less rounded form when at rest (Fig. 4).

In fixed and stained preparations the lymphocytes found on the surface of the large wandering cells and giant cells, and more rarely on the cover-slip, showed a greater diversity of form than the living ones in the plasma clot (Figs. 8 to 10). This was probably due to the peculiarities of the environment during life, where they had a more or less flat surface to which they could adhere and upon which they could spread out.

Neither mitosis nor amitosis was observed in lymphocytes. All the lymphocytes studied were mononuclear.

#### SUMMARY.

1. Lymphocytes were usually the first cells to migrate out into the plasmic clot from explanted pieces of lymph nodes.

2. Their paths of migration were irregular but in general they proceeded away from the explant.

3. The lymphocytes migrated at rates varying from 0.03 to 0.0013 mm. per minute. The rate of any one varied from minute to minute, and they often came to rest for varying lengths of time.

4. The migrating lymphocytes were very much elongated, with the nucleus always near the anterior end. The elongated tail contained the endoplasm with a few granular mitochondria and usually a few granules which took up neutral red.

5. The lymphocytes in cultures made from normal and pathological lymph nodes in auto- and homoplasma showed no differences.

## EXPLANATION OF PLATES.

## PLATE 10.

FIG. 1. 48 hour culture from a metastatic sarcomatous node in homoplasma. A migrating lymphocyte (No. 23, Table I) at  $\frac{1}{2}$  minute intervals. The change from the migrating form to the resting form and back to the migrating is shown. Distance traversed during the minute 0.025 mm.  $\times 1,250$ .

FIG. 2. 20 hour culture from a normal node in homoplasma. A migrating lymphocyte (No. 18, Table I) drawn at 1 minute intervals for 1 hour. The broken arrow points to the explant.  $\times 750$ .

FIG. 3. 24 hour culture from a lymphadenitis specimen in homoplasma. Migrating lymphocytes in the same field drawn at 1 minute intervals.  $\times 750$ .

FIG. 4.  $1\frac{1}{2}$  hour culture from a metastatic carcinomatous node in homoplasma. A migrating lymphocyte (No. 1, Table I) and a large lymphocyte drawn at 1 minute intervals.  $\times 750$ .

FIG. 5. 4 day culture from tuberculous node in autoplasm. Four migrating lymphocytes in the same field (Nos. 28, 29, 30, and 31, Table I) drawn at 1 minute intervals.  $\times 750$ .

## PLATE 11.

FIG. 6. 7 day culture from a normal node in autoplasm. Four resting lymphocytes and two large wandering cells on the cover-slip. Neutral red and Janus green were added at the time of observation. The lymphocytes showed neutral red granules and green mitochondrial granules. The large wandering cells had a deep red center (dark granular in the figure) near the nucleus, surrounded by an extensive area of fat globules. Mitochondria were abundant about the fat area and extended into the clear ectoplasm.  $\times 750$ .

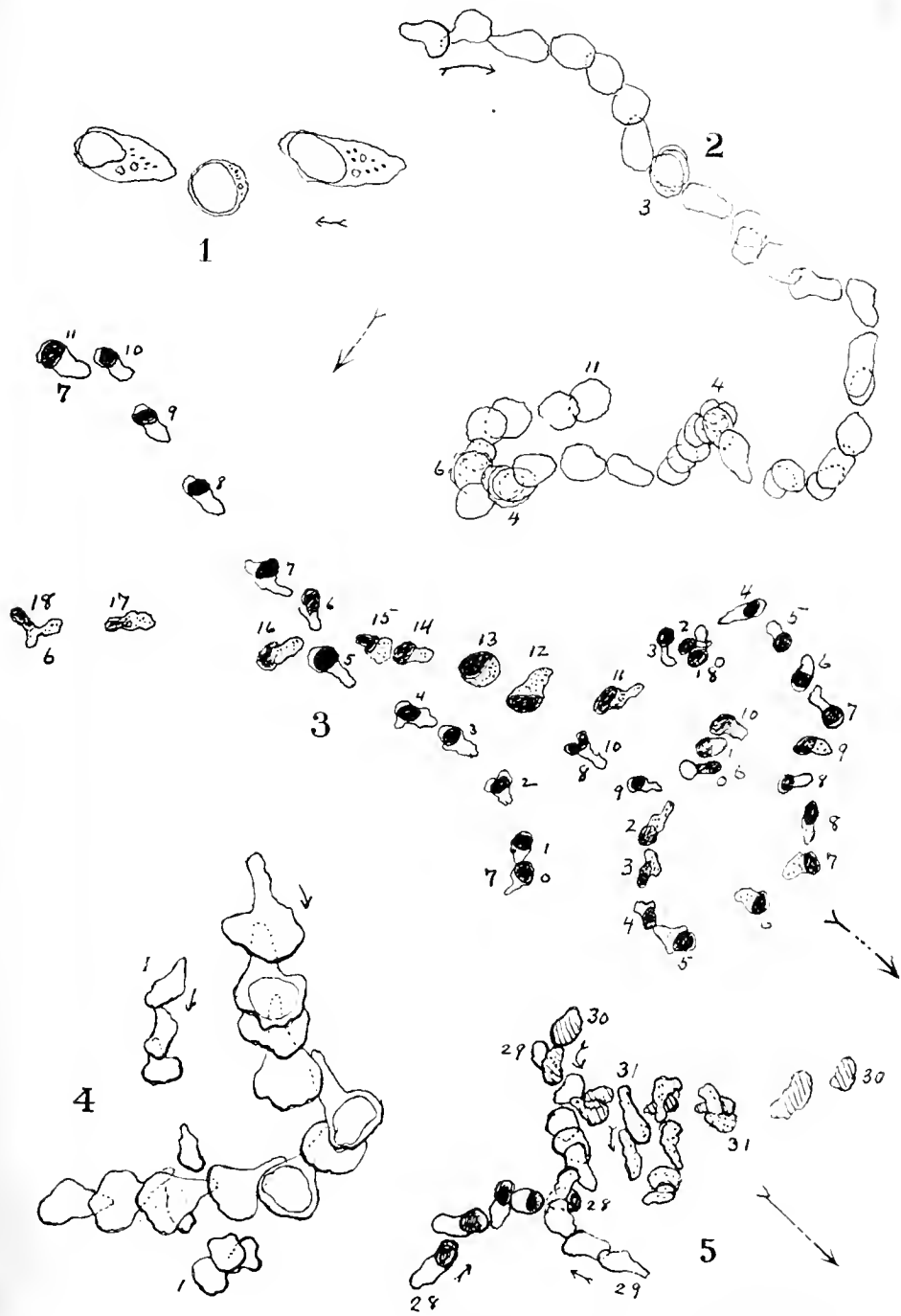
FIG. 7. 96 hour culture from a metastatic sarcomatous node in homoplasma. Two resting lymphocytes showing neutral red granules. Neutral red was added at the time of observation.  $\times 1,250$ .

FIG. 8. Fixed and stained specimen from a 48 hour culture of a tuberculous node in homoplasma. Five migrating lymphocytes are shown on the surface of a large mononuclear wandering cell which is spread out on the surface of the cover-slip. The wandering cell contains dead lymphocytes in various stages of digestion.  $\times 1,250$ .

FIG. 9. Five migrating lymphocytes on the surface of another large wandering cell from the same culture as Fig. 8.  $\times 1,100$ .

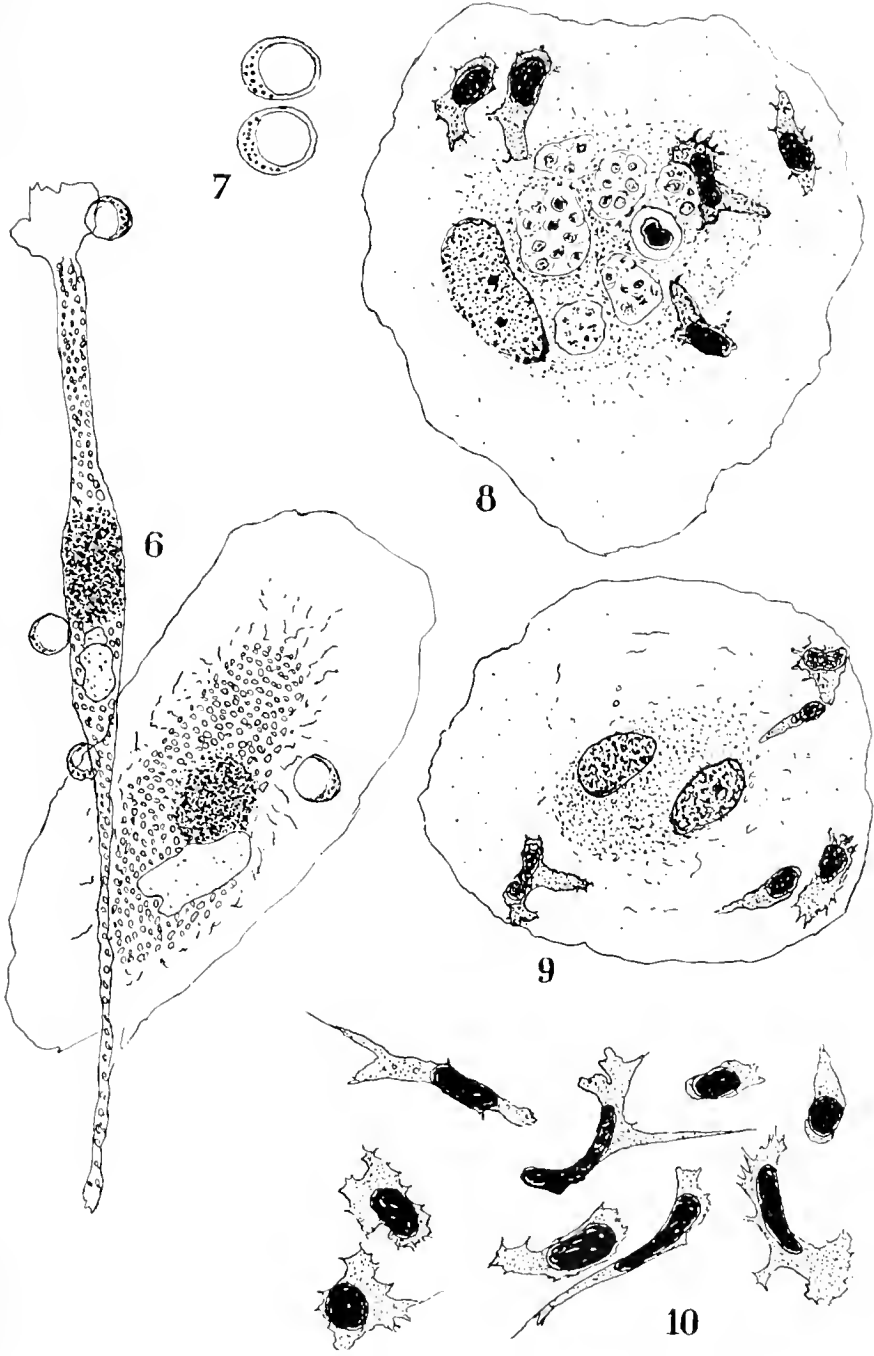
FIG. 10. Nine migrating lymphocytes from the surface of other wandering cells from the same culture as Fig. 8.  $\times 1,250$ .





(Lewis and Webster: Plasma cultures of human cells)





(Lewis and Webster: Plasma cultures of human lymph nodes.)





## STUDIES ON ENDOTHELIAL REACTIONS.

### IV. THE ENDOTHELIUM IN EXPERIMENTAL GENERAL MILIARY TUBERCULOSIS IN RABBITS.

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PLATE 12.

(Received for publication, September 21, 1920.)

It is the purpose of this paper and the others of the series (Foot, 1919, 1920, *a, b*) to draw attention to the important part taken by cells, originating in capillary endothelium, in various types of inflammation. In the first paper (Foot, 1919) their reaction to the presence of a foreign body (agar-agar) was discussed; in the two succeeding their behavior in experimental subcutaneous and pulmonary tuberculosis in the rabbit was described. By means of intravenous injections of a colloidal suspension of carbon it was shown that the cells forming the foreign body tubercles, as well as those found in lesions of experimental tuberculosis, were traceable to the endothelium of the neighboring capillaries. The present paper is a report of the histogenesis of the cells forming the tubercles produced in rabbits by the intravenous injection of tubercle bacilli. Such a general infection as this simultaneously produces lesions in various organs, and the question arises whether the cells found in all these are traceable to the capillary endothelium.

The literature on experimental tuberculosis has already been discussed at length in the earlier articles of the series (Foot, 1920, *a, b*), but it may be well to review it briefly. Sewell, Watanabe, and Wechsberg have experimented upon the lung, studying the lesions produced by the insufflation of tubercle bacilli through the trachea, with or without the use of a vital staining method.<sup>1</sup> The

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<sup>1</sup> A recent article by Permar (Permar, H. H., *J. Med. Research*, 1920, xlii, 9) has appeared since the present paper was submitted for publication. It is interesting to compare his findings in experiments on intratracheal injections of carmine in guinea pigs and rabbits, coincident with intravenous administration of isamine blue, with those of this paper and of Paper III (Foot, 1920, *b*). His conclusions as to the endothelial origin of the phagocytic cells of the lung tissue coincide in every way with mine and his emphasis of the non-participation of the epithelium in these phenomena of phagocytosis seems well supported by facts.

liver has been extensively studied because of the readiness with which exact amounts of bacilli can be introduced into the intramesenteric circulation and thence into the portal vein and its radicles in the liver. Among others Kockel, Miller (1902), Oppenheimer, Goldmann, Evans, Bowman, and Winternitz, Kiyono, and more recently Soper have investigated the histogenesis of tubercles produced by the intraportal injection of tubercle bacilli. Kostenitsch and Wolkow in 1892 studied tuberculous lesions produced in the cornea and kidney of the rabbit by means of local injections. So far as can be ascertained there are no reports on experimental work on miliary tuberculosis, in which methods of vital staining have been brought to bear.

Since the consensus of opinion has not been in favor of the endothelial origin of the so called epithelioid cell, particularly in the case of the lung and kidney, the following experiments were performed which confirm the theory that the tubercle in the lung and kidney is not of epithelial origin.

Two vital stains were employed, one a benzidine dye—Niagara blue 3 B<sup>2</sup>—the other a colloidal suspension of carbon in the form of Higgins' waterproof India ink, as in the preceding experiments. This is similar to the suspension devised by McJunkin and more easily prepared. The former gives a granular vital staining reaction, quite similar to that obtained with trypan blue, in the macrophages of Metschnikoff (a class of phagocytes designated by a large variety of names, but undoubtedly of endothelial origin), the lymphoid reticulum, the endothelium of the liver sinusoids, which is known as Kupffer cells, the fibroblasts, and the epithelium of the convoluted tubules of the kidney and, to a slight degree, that of the liver. It also appears in the polymorphonuclear neutrophils under conditions of extravascular existence or circulatory stagnation. Necrotic tissue and occasionally elastic fibers take on a diffuse blue color in its presence. Higgins' ink is chiefly taken up *in situ* by the endothelium of the smaller capillaries in certain organs, but it is also phagocytosed by the endothelium anywhere in the presence of inflammation. The free endothelial phagocytes likewise take it up in large quantities; if a benzidine dye is also present granules of carbon and this dye are taken up by these cells at the same time.

<sup>2</sup> The Niagara blue 3 B was kindly furnished by Dr. George B. Wislocki, of the Department of Surgery of Harvard Medical School.

Therefore in an experimentally produced miliary tuberculosis in which both of these dyes are present, the epithelioid cells will contain ink only if they are the product of the only tissue that takes it up; that is, the endothelium. It was of particular interest to determine whether this would be true in the lesions produced in the spleen, lymph nodes, and omentum, where many phagocytes are present and where the reticulum, on the one hand, and the mesothelium, on the other, have been considered the parent tissues of the epithelioid cells. It would be well to know also whether or not the renal epithelium contributes to the tubercles in the kidney, as claimed by Kostenitsch and Wolkow.

By using the two dyes together it is possible to obtain a relatively exact differential staining reaction; the macrophages, lymphoid reticulo-endothelial cells, and the mesothelium, and the convoluted tubules of the kidney show a marked affinity for Niagara blue. Although many of these cells take up ink the renal tubular epithelium never does so, and the others always show more blue than black granules in the presence of both dyes.

Soper has quoted the experience of other observers, as well as himself, to show that the epithelioid cell loses its affinity for benzidine dyes once it has taken up tubercle bacilli, or has come under their influence. In studying the formation of subcutaneous tubercles I (1920, *a*) have found this to be in a measure true, as the cells seem to become vacuolated and the dye granules dispersed and lost after tubercle bacilli are phagocytosed. It does not seem that cells loaded with these dyes, for example lymphoid reticular cells or omental macrophages, would immediately lose all the dye in the presence of tubercle bacilli, nor do the findings of this experiment indicate that such is the case.

Rabbits have, therefore, been injected intravenously with bovine tubercle bacilli and at the same time been vitally stained, the resulting lesions being studied to determine the origin not only of their epithelioid cells, but also of the so called tubercle reticulum. By combining the vital and the usual connective tissue stains it is hoped that new light may be shed on the origin of this fibrillar structure, particularly by observing its formation in organs normally poor in collagen fibrils. Kon, and Yoshida, working with Rössle, and more recently Warren have demonstrated the possibility of its being produced by the endothelium. Downey has discussed the literature on this

subject in connection with the formation of the fibrils of the lymphoid reticulum. Hueck's recent communication further strengthens this view which is discussed more fully below.

### *Technique.*

Four rabbits were given intraperitoneally 10 cc. of a 1 per cent solution of Niagara blue 3 B (twice purified) in distilled water. The next day this injection was repeated, with a 1 per cent solution of the commercially pure dye, which is less concentrated. The 3rd day the injection with the weaker dye was repeated and 5 cc. of Higgins' waterproof ink and distilled water, in equal parts, were introduced into an ear vein. The animals were now, by this means, vitally stained; their skin and mucosa were a deep ultramarine. On the 4th day each was given 1 cc. of a suspension of bovine tubercle bacilli intravenously, the strain employed being one of those used and tested out in previous experiments (Bov. XIV), and the dilution such that 1 cc. equaled 1 mg. of the bacilli. The suspension was made by rubbing up a weighed amount of culture from glycerol agar slants in normal salt solution, by means of a glass bulb pestle, shaking in a machine, and diluting and rediluting with normal saline solution until the desired concentration was reached. Intraperitoneal injections of 10 cc. of 1 per cent twice purified Niagara blue and intravenous injections of 5 cc. of 50 per cent Higgins' ink were instituted 3 days later and administered twice a week until the animals died or were killed. This kept all the rabbits vitally stained, with one exception. Rabbit 1 was killed 7 weeks after the experiment was begun, but the injections were only continued for 4 weeks, in this instance, in order to see what would become of the dyes accumulated in the tissues during that time. Later two other rabbits were given one intravenous injection of 1 mg. of the same strain of tubercle bacilli and the dyes were withheld until the 3rd week, when they were administered in exactly the same way as in the first set and continued until the animals were killed. The object was to begin supplying the dyes to the tissues at the approximate onset of the disease, instead of simultaneously with the inoculation.

This lot of six rabbits gives a series of potential cases of miliary tuberculosis representing stages of 1, 2, 2½, 4, 5, and 7 weeks development. All except one were killed by injecting 4 per cent neutral formaldehyde into the beating heart under anesthesia. Rabbit 2 died of an intercurrent coccal infection after 2 weeks, but showed a few pulmonary tubercles. The tissues were fixed both in neutral 4 per cent formaldehyde and in Helly's fluid (potassium bichromate 2.5 gm., corrosive sublimate 7 gm., water 100 cc., and neutral 40 per cent formaldehyde 10 cc.) in order to compare the action of these fixatives on the intracellular granules of Niagara blue. Helly's fluid is found to be in every way superior to neutral formaldehyde alone; the granules are somewhat paler than when only formaldehyde is used, but as they are apt to be very dark in the latter case, this is a distinct advantage as it enhances the blue color and prevents confusion between

this dye and the ink. Sodium hyposulfite should not be used to remove the iodine, before the final staining, as it tends to bleach the dye completely; 95 per cent alcohol, although slower, is preferable, as it does not affect the vital stain in any way.

Sections of 5 microns were cut in paraffin and stained with Mayer's aqueous carmalum (ammonia alum 20 gm., carminic acid 2 gm., and distilled water 400 cc.), with Mallory's phosphotungstic acid-hematoxylin and aniline blue connective tissue stain, and with Delafield's hematoxylin in conjunction with the Ziehl-Neelsen carbolfuchsin stain. It is, of course, necessary to rely upon a red nuclear stain for the routine procedure, in order to get the best contrasts with the blue vital dye; there is too little difference in color between this and hematoxylin or methylene blue to render them available except for topography or for photographic purposes. Sections were also impregnated by the Bielschowsky-Maresch method. Six organs were chosen for detailed study as described below.

### *Pulmonary Lesions.*

Since bacilli, after being introduced into the circulation, are first swept into the pulmonary capillaries and, as Miller (1919-20) has shown, into the intrapulmonary lymph nodules, lesions are found developing in this organ before they can be found elsewhere. After 1 week two types of tubercle are distinguishable. The first consists of small aggregations of deeply ink-stained cells, 70 microns in diameter, which in the light of their further development, seem to be merely an agglomeration of phagocytic cells in response to the presence of the ink. The second type is composed of true, specific tubercles, few in number and only found after some searching. They show no Niagara blue, but their epithelioid cells contain ink globules. After 17 days development larger tubercles, about 375 microns in diameter, can be found. They show a few syncytia, or giant cells, and contain a uniform amount of ink in the cells composing them. Polymorphonuclear leucocytes now appear at the periphery of the tubercles. The latter are obviously interstitial in origin and are situated near the blood vessels of larger caliber; one occasionally finds crescentic thickenings of the endothelium of these vessels, the crescents being deeply impregnated with ink globules. Similar lesions, which are not crescentic, are found in the intrapulmonary lymph nodules, near bronchioles. The alveolar spaces are not involved until surrounded by the tuberculous infiltrate, when they begin to fill up with the large, emigrated endothelial cells described in the preceding paper (Foot,

1920, b). These cells, like those of the tubercles, contain fine granules of carbon.

After 4 weeks development the tubercles are visible to the naked eye, being 0.3 to 0.7 mm. in diameter and occupying the walls and air sacs of the lobules affected. The tubercles often include larger vessels and cause their obliteration by overgrowth of the lining endothelium, which proliferates not only into the crescentic masses just described, but becomes a plug of anastomosing, rather stellate cells, not unlike the embryonal mesenchyma in appearance. Whether these lesions start within the vessels and work outward, or whether they surround and involve the vessels from without, is sometimes difficult to determine. As the infection is presumably intravascular, in this experiment, the first hypothesis is the more plausible. All the cells composing the tubercles are dotted with ink globules and a few now show granules of Niagara blue. Caseation begins at the center of the larger lesions, the caseous mass taking on a diffuse bluish stain. Polymorphonuclear leucocytes penetrating the tubercles also show a granular blue vital stain, but no ink. This indicates that the blue dye is available here, as elsewhere in the body and that it has no particular affinity for the epithelioid cells, whereas the ink has. As in the experiments on intratracheal infection no activity is seen among the epithelial cells, other than regenerative phenomena. A light vermilion blush now suffuses the tubercles in the Van Gieson preparations, while a similarly faint bluish tinge appears in those stained with Mallory's connective tissue method. This can be seen to be due to the appearance of delicate fibrils in the lesions, not only among fibroblasts but also in and between the ink-containing epithelioid cells. These fibrillæ are also demonstrable as pinkish threads with the phosphotungstic acid-hematoxylin stain. While they are always red with the Van Gieson process, they may be red or reddish with Mallory's connective tissue stain, or blue or purplish with the phosphotungstic acid-hematoxylin. This would, presumably, indicate that they were gradually becoming impregnated with collagenous material.

After 5 weeks all these processes are more striking. The fibrous reticulum is now well formed and easily seen, the caseation more advanced and a deeper blue. After 7 weeks the lung is so widely

involved as to leave little normal tissue. In this case, as noted under Technique, the use of the dyes was discontinued in the 4th week; consequently the newer tubercles are almost devoid of ink and Niagara blue, while the older ones show plenty of both, the latter in diffuse form in the caseous areas. Older tubercles are sometimes centrally stained and peripherally free from the vital dye, the younger portions of the lesion having apparently formed after the supply of available dye was exhausted. There are a great many intravascular, crescentic, and deeply ink-stained tubercles in the sections from this rabbit, as well as a few of the obliterating, retiform type.

Thus it is found in the lung that the tubercles develop in and near larger vessels, that they contain much ink, but little granular Niagara blue, and that the reticulum is formed chiefly in the epithelioid cells. Groups of migrating endothelial cells lying free in the alveoli can be seen in the 5 and 7 week preparations, in which delicate fibrils are demonstrable. There is no question of any relation between these cells and the alveolar walls, as their origin has been worked out and reported in the preceding paper (Foot, 1920, *b*). The fibrils first form about the periphery of the cells by consolidation of their cytoplasm and cell processes (ectoplasm); next there is vacuolation of the endoplasm and a consolidation of the walls of the vacuoles, with the production of fibrillar circles and lines, which gradually seem to twist and break into fibrillæ. The process is similar to that described by Hueck in his article on the mesenchyma. This is not strange in view of the embryonal origin of these cells.

Sections from the lung after 5 weeks were impregnated with silver by the Bielschowsky-Maresch method with the view of obtaining an idea of the distribution of the reticulum fibers among the epithelioid cells. Fig. 1 shows that they are readily demonstrable in cells that contain carbon particles. The majority of the epithelioid cells were found to be free from reticulum fibers, but there are many places in the sections where the fibers can be found running in and among these cells and forming a fairly close network about them. Of course, many fibers run out from the alveolar walls into the exudate, but there are instances in which no connection between these and the smaller fibrils in the epithelioid cells is demonstrable. This method of impregnation, then, bears out what has already been noted with the connective tissue stains.

*Hepatic Lesions.*

In the hepatic lesions also, distinction should be made between small pseudotubercles, apparently due to the presence of ink, and the true tubercles resulting from the infection. During the first 3 weeks there are slight changes in the sinusoidal endothelium, in part probably due to the ink, in part possibly the forerunner of true tubercles; but it is not until the 4th week that fully developed, specific lesions appear. They seem to begin as syncytia and to be situated in sinusoids lying in the mid-zone, between the hepatic and portal systems, usually nearer the former. The cells forming them are so heavily laden with ink globules that it is difficult to make out their histology (Fig. 2). The ink is usually arranged peripherally in the syncytia and single cells, forming a dense black rim that occupies much the same portion of the cytoplasm in the former, as that in which the nuclei are generally grouped, while in the latter it forms a ring about the single nucleus. In no organ is it so plentiful in the cells as in the liver. There is no indication that the syncytia are formed by mitotic nuclear division, without cytoplasmic division, as claimed by Soper; it seems more likely that they are the result of a fusion of several single cells. This is the rule in the subcutaneous lesions, and there is no apparent reason why there should be an exception in the liver endothelium. After 5 weeks the tubercle reticulum is demonstrable in the sections stained by appropriate methods. The fibers are best seen if green or greenish brown filters are interposed between the condenser and slide, when they stand out sharply. Here, as in the lung, they can be definitely demonstrated in the epithelioid cells, as well as in the few connective tissue cells present.

*Splenic Lesions.*

In the preparations representing the first few weeks development nothing definite can be distinguished. The pulp and its phagocytes are intensely blackened with ink, and the splenic reticular cells are deeply stained with the blue dye, taking up the ink to a much less degree. In the 4 week preparations the tubercles are mature and situated at the periphery or near the center of the Malpighian corpuscles. Their epithelioid cells contain a varying amount of ink



but far less than heretofore encountered in experimental tubercles of this type. Only a few of them contain Niagara blue granules. Small tubercles forming in the secondary nodules of Flemming show much ink in their cells. The tubercle reticulum is beginning to form.

After 5 weeks, caseation is found and the caseous material is stained a diffuse blue. There is the same scanty deposit of ink in the tubercles (Fig. 3) of the Malpighian corpuscles but a moderate amount in the secondary nodules. Although the splenic reticular cells are deep blue, only a few of the epithelioid cells show this stain. After 7 weeks, however, the tubercles are deeply stained with ink. Besides the tubercles, one finds groups of ink-bearing cells in the lymphoid nodules; they show no tubercle reticulum, while the true tubercles exhibit a fully formed network of fibrils. In this way the two are readily distinguishable. The splenic sinuses are widely dilated and almost free from ink-bearing cells; it seems as if these had migrated to the lymphoid tissue and, possibly, into the tubercles as well. The reticular cells of the splenic tissue are still deeply stained with Niagara blue.

Thus, although the splenic sinuses and their free cells are so heavily laden with ink whenever it is administered intravenously, relatively little of the ink seems to be found in the cells forming the tubercles located in the lymphoid tissue of the Malpighian corpuscles, while a moderate amount is present in the smaller, or secondary nodules. This is not readily explained; the subject will be discussed further in the consideration of the lesions in the lymph nodes, where a somewhat similar condition exists.

#### *Renal Lesions.*

Definite tubercles are first found in the sections from the 4 week rabbit although there are indefinite changes in the glomerular tufts and the walls of the vasa recta before this time. The tubercles vary from 75 to 750 microns in size and appear to be of two types, a moderately diffuse infiltration of the interstitial tissue between the cortical tubules, and more localized collections of epithelioid cells, with an underlying glomerular topography—one can make out the remains of Bowman's capsule with swollen cells surrounded by the tuberculous lesion. In the first type, there are many small endothelial

leucocytes, similar to those seen in the subcutaneous lesions described in the first and second papers of this series (Foot, 1919, 1920, *a*). These are compact cells, with a bean-shaped nucleus and rather dense cytoplasm, about half the size of fully developed epithelioid cells, many of which are found in these tubercles. These cells often contain ink globules, as do their syncytial derivatives. The tubular epithelium, on the other hand, takes up no ink, although that of the proximal convoluted tubules is vitally stained with Niagara blue. It is significant that the tubercles show ink and that their cells take on no blue stain, except in scattered places where degeneration is obvious. One can find mitotic figures in the epithelium of the surrounded tubules, but this is probably an attempt at regeneration, as they are found only in cells that are still *in situ* in the tubules. In some of these diffuse tubercles it is possible to see the tubular epithelium degenerating and disintegrating, with endothelial cells, containing ink, pressing in upon its thinned out portions and apparently producing pressure atrophy.

After 5 weeks the tubercles are more numerous, larger, and show beginning central necrosis, with the usual diffuse blue stain in the caseous areas. The two types of tubercles, glomerular and interstitial, can still be distinguished. Whereas the tubercles were formerly all cortical, there are now a few of the interstitial type among the collecting tubules and Henle's loops in the medulla. They do not differ from the cortical interstitial type and their cells show ink (Fig. 4), but no Niagara blue. After 7 weeks the picture is not noticeably altered, but is merely more pronounced. The tubercle reticulum is demonstrable after the 4th week and is best seen in the 7 week lesions, differing in no way from that of the lesions in other organs.

That many of the tubercles originate in the glomeruli is indicated by the following facts. (1) Remains of glomerular structure are present in some of them. (2) The early tubercles are often rounded and of the same diameter as the average glomerulus. (3) The tubercles occur chiefly in the cortex. (4) The glomerulus is the chief renal structure showing ink in its cells; moreover, there is none in the tubules. Nevertheless, there are other lesions showing no evidence of glomerular architecture which occur near the vasa recta and may be found well down in the pyramids where no glomeruli are located. Ink is found in the endothelium of the vasa recta in controls. It

seems probable, then, that the tubercles originate from two sources, the endothelium of the glomerular capillaries and that of the capillary branches of the vasa recta, the former remaining near their site of origin, the latter migrating into the interstitial connective tissue between the tubules. There is no evidence that the epithelium takes an active, formative part in the process, although a few epithelial cells may be included in the tubercles and survive there for a time. Such cells, which have their origin in the vitally stained convoluted tubules, may contain granules of Niagara blue, and their nuclei often stain a bright blue, which is pathognomonic of degeneration and indicates early karyolysis. The ink globules prove to be an invaluable indicator of the origin of the component cells of the tubercles in the kidney as elsewhere.

#### *Omental Lesions.*

The omentum, bathed as it is in a copious supply of Niagara blue from the intraperitoneal injections, shows deeply stained mesothelial cells on the surface, some staining of the reticulum of the taches laiteuses, and a double staining of the wandering macrophages in its meshes. These macrophages contain some ink as well as the blue granules of the benzidine dye. The vascular endothelium of the complex capillary network, however, is dotted with black spots alone. The tubercles appear early, the first being found in the 17 day specimens, beginning as single syncytia and gradually enlarging as time goes on, until miliary tubercles are formed. The simplest of these lie within and not on the omentum, between the peritoneal layers; their syncytia contain ink alone. After 4 weeks they are, of course, more abundant, and the ink still predominates, although a few blue granules are demonstrable in some of the single cells near the periphery which are doubtless wandering macrophages. The taches laiteuses are free from ink and from tubercles. After 5 weeks tubercles can be found penetrating the peritoneal covering, which is broken through and shows dissociated and swollen cells. The general impression obtained is similar to that gained from a study of an ulcer of the intestine; the mesothelial cells are apt to be thickened and piled up at the edges of the lesion, which is composed of larger, paler cells

containing ink and forming a mass that extends well into the connective tissue of the omentum. The mesothelial cells are blue-stained and readily distinguishable from the epithelioid cells as long as they remain well preserved; once they begin to degenerate they are more difficult to distinguish, as they lose the blue granules. It seems probable, from what has just been described, that the tubercles here are formed primarily from the endothelium of the omental capillaries, near which they are first found. The macrophages probably join in the process as it progresses, while the mesothelial tissue appears to be, at the most, sympathetically involved.

#### *Lesions in Lymph Nodes.*

Enlarged lymph nodes were regularly found in the axillæ and groins of the rabbits, as well as the peribronchial groups, and were always excised and examined. During the first 3 weeks the medulla is very edematous and its sinuses are filled with large cells of the macrophage type which are deeply stained with Niagara blue and contain phagocytosed cellular debris. They increase in number and size and, in the 17 day specimens, fuse to form free lying syncytia. They do not form tubercles, and carbolfuchsin shows them to be filled with short, plump, reddish rods, totally unlike tubercle bacilli and possibly representing mitochondria. The lymphoid reticulum is stained deeply with Niagara blue, and there is no ink present, except in or near vessels of the cortical nodules.

Tubercles are first found in the 4 week specimens. They are composed of large, pale, anastomosing cells with an evenly distributed amount of carbon granules, which are very fine, but still perfectly evident (Fig. 5—after 5 weeks). Small dilated capillaries are usually demonstrable in their immediate vicinity, five with ink in the endothelium and blood corpuscles in the lumina being found at the periphery of one such tubercle. The tubercle reticulum is suggested in the 4 week specimens, and is well formed and easily demonstrable in the 5 and 7 week preparations. Although the lymphoid reticulum is everywhere deeply stained with Niagara blue, little or none is found in the tubercles. In the 7 week preparations the medulla is nearly free from the large, free cells, and the tubercles show much more ink than those of the earlier stages.

Thus less carbon is deposited in the tubercles formed in the lymphoid tissue of the spleen and lymph nodes than in those of the other organs, with the exception of the kidney. This is probably due to the fact that these tissues have little affinity for carbon and, in the case of the lymphoid tissue, are poorly supplied with blood capillaries. Where the latter abound, plenty of ink is found, as in the lungs, liver, and splenic pulp. It is interesting that, in both spleen and lymph nodes, the large, free lying phagocytes of the pulp or medulla appear to migrate into the lymphoid tissue, for as soon as the administration of the dyes ceases, they leave the sinuses, while the lymphoid tissue becomes full of carbon-bearing cells. That the blue granules are not seen in the lymph nodules in this case is possibly due to the fact that they are more diffusible and can be excreted by the cells that contain them and then be eliminated through the blood and urine, the latter always being of a bluish green color. The carbon seems to be more difficult to remove and is probably retained until the disintegration of the cells containing it sets it free for other phagocytes to take up.

#### DISCUSSION AND SUMMARY.

A review of the findings in this series of experimental inoculations brings out clearly two points: (1) the specific lesion of the miliary type is composed chiefly of cells of endothelial origin, apparently coming, for the most part, from the walls of the small capillaries near it; and (2) these cells are capable of forming the reticulum of the tubercles and hence collagen. In Hueck's paper, which has already been referred to, the origin and development of the vascular systems are discussed as a subdivision of the mesenchyma. Hueck states that he considers mesenchymal cells of the endothelial type capable of forming collagen fibrils, even in adult life, but indicates that he would limit this attribute more or less strictly to the endothelium of the capillaries. He points out that no "silver lines" can be demonstrated in the capillary endothelium, while they are readily shown in the endothelium of the larger, more specialized vessels; the capillary endothelium is more syncytial, less differentiated, and hence capable of assuming rather varied forms. Hueck's conclusions are in agreement with the findings of the present paper which emphasizes a point that

has been stressed in each of the reports of the series; namely, the importance of the capillary endothelium as distinguished from the endothelium in general.

Rössle and Yoshida several years ago claimed that endothelial cells could produce collagen fibrils by a process of metaplasia. It seems unnecessary, in the light of what has just been said, to apply the term metaplasia to this phenomenon. These cells evidently represent a cytological element in the body that is intended for the process of repair and is unusually plastic, being able to assume varied functions. It is, as it were, a persisting mesenchyma; if this was not so and there were no polyblastic cells present, the process would be slower and less effective. Hueck's ideas on the impregnation of "indifferent fibrillæ" with collagen, elacin, or other substances are also in accord with the staining phenomena observed in the formation of the tubercle reticulum. There is at first a fibrillar or retiform structure to the cytoplasm of the epithelioid cells, without a definite collagen staining reaction. The fibrils can be seen but their color at first does not differ materially from that of the cytoplasm. Gradually, however, they take on a faint pink stain with acid fuchsin or phosphotungstic acid-hematoxylin, or a faint turquoise-blue with aniline blue. These colors increase steadily in intensity as the fibrils become thicker and more evident. The changes from black to brown in the Bielschowsky-Maresch technique are analogous.

That Niagara blue does not appear in the epithelioid cells to any extent must mean one of two things, either the cells do not take it up, or they lose it as soon as they differentiate from the more compact to the larger, paler type. It is certain that they take up and retain the ink through all their phases; it seems doubtful that they have lost many blue granules, as one can seldom demonstrate such a process. If such a reaction did occur the earliest specimens should show groups of cells containing blue granules, while the later stages would show progressive loss of this color. This does not seem to be the case, except in a few instances, when cells with blue granules are occasionally included in tubercles. They are then most often found near the caseous centers.

There may be some question as to the part played by the lymphoid reticular cells in tubercle formation in the lymphoid tissue. If

they do form the epithelioid cells, they must lose their charge of blue dye in so doing and acquire a heavier load of carbon granules. It would seem easier to infer that the epithelioid cells are derived from the endothelium of the scattered vascular capillaries. This would explain the absence of blue granules as well as the smaller amount of carbon present in comparison with that found in the epithelioid cells formed in organs more richly supplied with blood capillaries.

#### CONCLUSIONS.

1. Miliary tubercles produced in the lung, liver, spleen, kidney, lymph nodes, and omentum of the rabbit by hematogenous tuberculous infection are composed chiefly of cells originating in the capillary vascular endothelium.

2. These cells have a marked affinity for carbon in colloidal suspension, which makes their identification possible.

3. A benzidine dye like Niagara blue, while acting selectively on macrophages, lymphoid reticular cells, renal convoluted tubular epithelium, and free polymorphonuclear leucocytes, is not found in the tubercles to any appreciable extent, unless there is necrosis, when the staining is diffuse.

4. The tubercle reticulum is composed of fibrils resembling connective tissue fibroglia and collagen fibers in every way and produced not only by fibroblasts, but by the endothelial cells themselves, after these have migrated from the vessels, differentiated into epithelioid cells, and formed tubercles. This process is well advanced 5 weeks after inoculation.

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## EXPLANATION OF PLATE 12.

Magnification,  $\times$  about 350. Fig. 1 is from a paraffin section impregnated by the Bielschowsky-Maresch method; the rest are from phosphotungstic acid-hematoxylin preparations.

FIG. 1. Reticulum fibrils and carbon particles in the epithelioid cells of a 5 week tubercle in the lung. It will be noted that the fibrils run in the cytoplasm of cells liberally dotted with carbon.

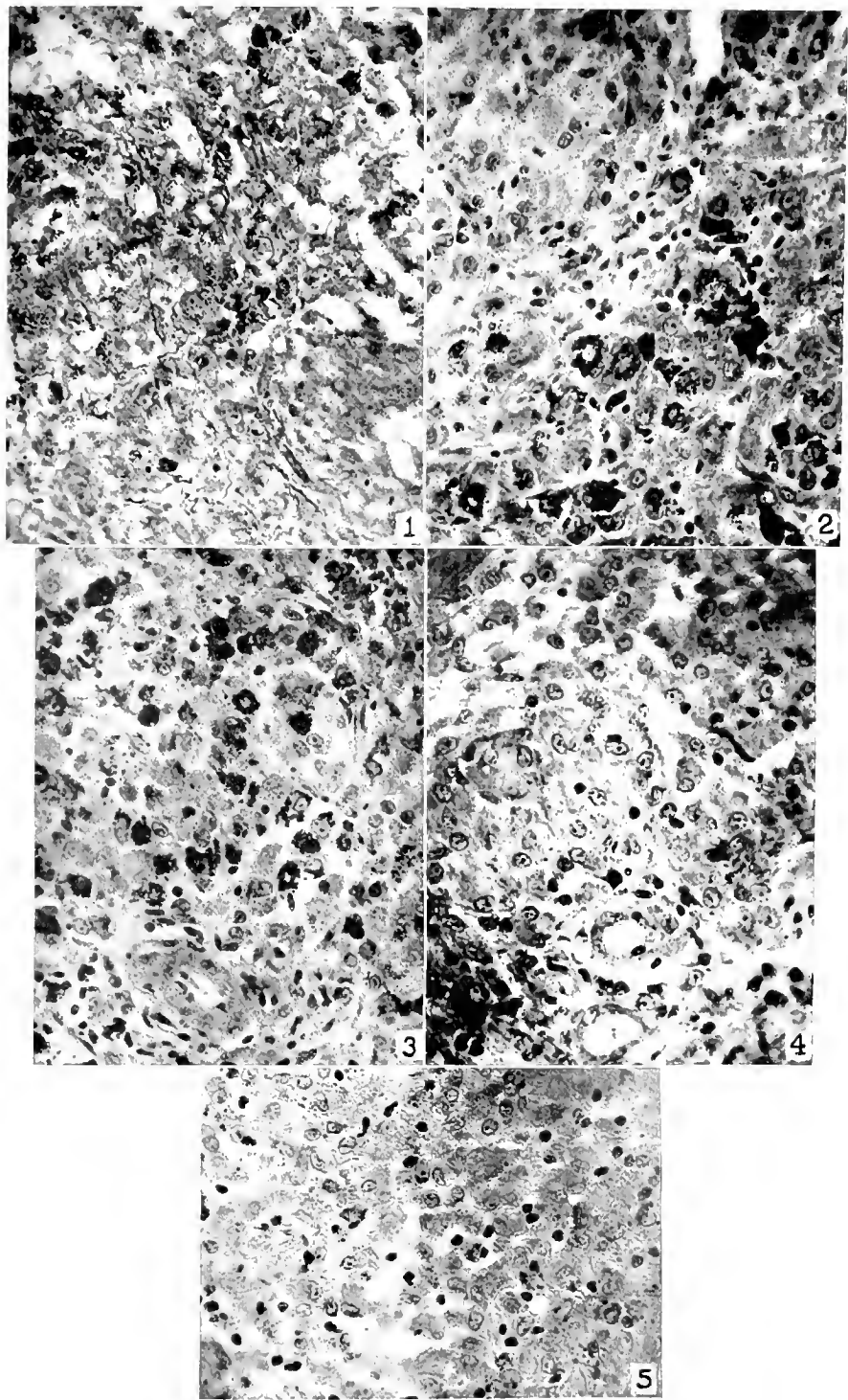
FIG. 2. A 5 week tubercle in the liver, showing the distribution of carbon particles and the peculiar ring-shaped masses referred to in the text.

FIG. 3. A 5 week tubercle in the spleen with more carbon present than is usual in such tubercles. Rings of ink particles are also found.

FIG. 4. A cortical, 5 week tubercle in the kidney. The carbon is rather sparsely distributed, but it can be made out clearly in many of the epithelioid cells.

FIG. 5. Tubercle in the same stage of development in a lymph node. The carbon is very finely divided and still more sparsely distributed, but several cells have a moderately heavy deposit, and careful search reveals several granules in most of the cells.





(Foot Endothelial reactions. IV.)



## CONDITION OF THE CAPILLARIES IN HISTAMINE SHOCK.

BY ARNOLD RICE RICH, M.D.

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PLATES 13 TO 17

(Received for publication, November 5, 1920.)

The recent work of Dale, Laidlaw, and Richards has brought into prominence the idea that the phenomena observed in shock may have their origin in a primary peripheral circulatory failure. These investigators have used the discovery that histamine ( $\beta$ -iminazolyethylamine) injected intravenously in minute quantities brings about promptly, in certain animals, a condition which is strikingly similar to and apparently identical with the clinical condition designated by the term shock. The blood pressure falls to a very low level and remains there; the pulse becomes small and feeble; the respirations are shallow, irregular, or gasping; the superficial reflexes are active, but a dulling of the sensibility permits the discontinuance of the anesthetic without discomfort to the animal which lies quietly in an apathetic state. Further points of resemblance between histamine shock and clinical shock can be found in detail in the papers by these workers.

The amino-acid histidine enters into the composition of practically every body protein. Since histamine is derived from this amino-acid by decarboxylation, there arises immediately the idea that histamine, or similar substances, may be formed in the body under certain conditions, especially following extensive destruction of tissues by trauma or by widespread inflammatory processes, and that the entry of such substances into the blood stream may be the fundamental cause of the shock which accompanies these conditions. At present, however, in spite of certain suggestive experimental evidence such ideas are entirely speculative, and the present work is not concerned with the question whether histamine and allied substances cause clinical shock. We are interested here rather in a study of the mode of action of histamine which confers upon it its shock-producing potentialities.

The action of histamine has been studied by Dale and his coworkers<sup>1,2</sup> who concluded that the base exerts upon all capillary endothelium a poisonous local effect which results in a dilatation of the lumen of the capillary and an increased permeability of the endothelial wall. The first of these effects is assumed to

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<sup>1</sup> Dale, H. H., and Richards, A. N., *J. Physiol.*, 1918-19, lii, 110.

<sup>2</sup> Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1918-19, lii, 355.

bring about an accumulation of blood within the capillary system and a consequent inadequate venous return to the heart (" . . . the shock-like failure of the circulation is due to accumulation of the blood in relaxed capillaries . . . the flow through the capillaries becomes ever slower and the tendency to stagnation greater, till ultimately only a relatively small part of the available blood is effectively circulating."<sup>2</sup> The increased permeability of the capillary walls is assumed to favor the escape of plasma from the vessels and so to impose an actual oligemia upon the already enfeebled circulation. While Dale and Laidlaw<sup>2</sup> are "convinced that general dilatation of the capillaries and loss of plasma from the blood are main factors in the production of the shock," they were unable to observe directly the reactions of capillaries subjected to the influence of histamine, but deduced their conclusions from other considerations, stating: "Several attempts were made to observe changes in the calibre of capillaries under a moderate power of the microscope, without any result to which we could attribute importance." Dale and Richards<sup>1</sup> had written previously: "We made several attempts to measure changes in the calibre of blood vessels—arterioles and capillaries—in the mesentery by direct observation with the microscope, histamine solution being directly applied to the area under observation. The results were not sufficiently convincing to ourselves to make it desirable to record them. Later experience of the ease with which this vasodilator action is impaired, by temporary interference with the circulation through an organ or exposure to cold, made it clear that satisfactory observations of this kind would need a specially elaborated technique."

Since this important theory of shock has as its basis the assumption of accumulation of the blood within a capillary system dilated by the action of an endothelial poison, the present work was begun, at the suggestion of Dr. W. G. MacCallum, in an attempt to study directly the effect of histamine upon the capillary system in order to determine (1) whether histamine is actually an endothelial poison acting locally upon capillaries to produce a dilatation of their lumina, (2) whether the capillary bed during histamine shock undergoes a general dilatation of a degree sufficient to endanger the circulation, and (3) whether such a dilatation precedes or follows the circulatory failure occurring in shock.

#### EXPERIMENTAL.

The omentum was chosen as the site most suitable for observation because its transparency favors microscopic study in fresh and fixed preparations and especially because the local action of histamine was

to be studied. Since the capillaries of the omentum are for the most part practically surface capillaries, they can easily be brought into contact with locally applied histamine solutions.

*Effect of Histamine Studied Microscopically in Vivo.*

An attempt was first made to study microscopically, *in vivo*, the effect of histamine upon the capillary circulation of the omentum.<sup>3</sup> Any satisfactory technique for such a study must safeguard the capillary bed under observation from at least four sources of error—trauma, mechanical interference with the circulation, temperature changes, and evaporation of moisture. In order to protect the field from abnormal temperature changes a box large enough to accommodate an anesthetized cat was constructed of wood, lined with asbestos, and equipped with a thermostat. The chamber was fitted with a glass window, a movable opening for the barrel of a microscope, and felt arm sleeves through which work could be carried on within the box while it was entirely closed to maintain a constant temperature. A microscope, from which the base and substage had been removed, was attached to a horizontal bar clamped to an upright rod so that it could be raised or lowered, advanced or retracted to the desired position. The stage was covered with a thick glass plate, and a minute electric globe taken from a small pocket flash-light was fitted beneath the stage and served for illumination; the amount of heat given off by this tiny lamp was too slight to be measured by an ordinary thermometer placed on the glass plate above it.

Dale and his coworkers found cats especially susceptible to the action of histamine. This was the animal used, therefore, in all the present experiments. A cat was anesthetized with ether, tracheotomized, and connection established with an ether respiration bottle by means of a tracheal cannula. The animal was then placed within the box in which the atmospheric temperature was maintained at about 37°C. The abdomen was opened along a midline incision, the edges of which were held apart by small Balfour retractors. The intestines were covered with pads soaked in normal saline solution; the glass stage was warmed to 37° C. by immersion in normal saline

<sup>3</sup> All operations were performed under ether anesthesia.

solution kept at that temperature. The microscope was now advanced and lowered so that the stage occupied a position within the abdominal opening on a level with the intestines. The omentum was then gently lifted onto the stage of the microscope, care being taken to avoid unnecessary stretching, twisting, pulling, or any manipulation which might traumatize the omentum or disturb its circulation even momentarily. The stage of the microscope was made to occupy such a position that the omentum could be lifted upon it with a minimal displacement from the normal situation. During the period of observation the omentum was continually kept moist by means of capillary pipettes from which normal saline solution flowed, warmed to body temperature. Thus the omentum lay upon the glass stage of the microscope scarcely disturbed from its normal position, was protected against the effects of drying, and its normal temperature was maintained. Throughout all the observations the omentum was handled as little as possible and always with extreme care.

With the low power objective the blood can be watched coursing through the capillaries and the smaller arterioles and venules of the omentum. Capillaries may easily be seen which have diameters so small that they permit the passage of only one red corpuscle at a time. The omentum may be shifted gently upon the stage until a capillary bed which is suitable for study comes into the field. A field which contained a small arteriole and venule, as well as a rich anastomosis of capillaries of different diameters, was usually chosen. Any individual vessel selected for observation was measured at intervals with an eyepiece micrometer in order that any variations in diameter might be detected. While the omentum lay undisturbed upon the stage of the microscope, capillaries were observed as long as 30 minutes, during which time there occurred no measurable changes in their diameters.

*Local Application of Histamine.*—After a field had been chosen for observation, the vessels were watched for several minutes in order to acquire an impression of the rate of flow through them, and the diameters of several selected capillaries were measured. Then, while the observer kept a careful watch over these measured capillaries and the blood stream flowing through them, an assistant, using a capillary pipette, flooded the field with a solution composed of his-

tamine<sup>4</sup> dissolved in normal saline solution warmed to body temperature. Solutions of histamine ranging in strength from 1:500,000 to 1:500 were in this manner applied locally to the capillaries of the omentum in a large number of experiments. Careful inspection and measurement disclosed no change whatever in the diameters of the capillaries or in the rate of flow through them, nor were any capillaries opened up which were not visible before the application of histamine. Under the conditions of these experiments histamine locally applied produced no detectable change in the appearance of the capillary circulation.

*Histamine Shock.*—In another series of experiments, while a field of capillaries was kept under observation, the animal was thrown into shock by an intravenous injection of 4 mg. of histamine, blood pressure tracings being taken from the carotid artery. As the blood pressure fell the rate of flow through the capillaries became slower, until the stream was moving in a strikingly sluggish manner; but again, as with local applications of histamine, no widening of the capillaries could be detected.

These experiments demonstrated that a pronounced slowing of the capillary stream accompanies the fall in blood pressure which histamine produces when injected intravenously. Two possibilities were suggested in explanation of the fact that the local application of histamine did not affect the size of the capillaries in these experiments. Either histamine exerts no effect upon the diameters of capillaries, or else the handling of the omentum necessary to bring it under the microscope, in spite of the care with which this was done, inflicted sufficient trauma to bring about either a capillary paralysis and dilatation or a loss of capillary irritability, with the result that the vessels could not be dilated further by the action of histamine or any other agent. Experiments were, therefore, carried out in which

<sup>4</sup> The histamine preparation used was the dihydrochloride of the base supplied by the Hoffmann La Roche Chemical Works of New York. The activity of the stock supply was tested from time to time upon the virgin uterus of the guinea pig and was found to produce the characteristic contractions at each test. The potency of this preparation was also exhibited in the numerous animal experiments performed during this study, in which it was used to induce shock. Fresh solutions were always prepared before every experiment.

irritants and alleged capillary dilators were applied locally to omenta under microscopic observation.

*Local Application of Inflammatory Agents.*—Among the substances used were cantharides, turpentine, croton oil, gold and sodium chloride, amyl nitrite, and chloroform. It is well known that irritants locally applied to the web of the frog's foot bring about a marked dilatation of the capillaries,<sup>5</sup> and that in mammals the intraperitoneal injection of these substances produces a dilatation of the peritoneal capillaries during the resulting inflammatory reaction. It was assumed, therefore, that, if the normal reactivity of the capillaries under observation in these experiments was undisturbed, the local application of such substances would be followed by definite changes in the capillary diameters. Some of the irritants brought about a stasis of blood in the capillary area to which they were applied—apparently a result of osmotic disturbances. In no instance was there seen any definite dilatation of the capillaries which, in some experiments, were observed as long as 30 minutes following the application.

*Effect of Histamine Studied in Fixed and Stained Preparations.*

Since the local application of such inflammatory agents failed to produce a detectable capillary dilatation, it seemed that the conditions of the experiment must have brought about some alteration of capillary irritability, and an attempt was made to determine what effect the handling of the omentum in these observations exerts upon the capillaries. In a series of experiments a midline incision was made in the abdomen of normal cats under anesthesia, and the abdominal cavity was immediately flooded with a fixative. The fixative used in all experiments was a modification of Zenker's fluid, formaldehyde being substituted for glacial acetic acid. The omentum was then quickly cut along the line of attachment and transferred to a dish of the fixative where it was left for 24 hours. It was washed, spread over large glass slides, and stained with hematoxylin

<sup>5</sup> The web of the frog's foot, while offering ordinarily a standard and convenient field for capillary observation, was unsuited for study in these experiments, because the frog is extremely resistant to the shock-producing effects of histamine.



and Van Gieson's stain. In such a preparation the arterioles, venules, and capillaries are distinguished in minute detail. Since the omentum was, in this manner, fixed before being subjected to any manipulation or exposure, these slides were assumed to reveal the normal appearance of the omental capillaries. In another series of experiments, after the abdomen had been opened, the omentum was lifted to the stage of the microscope with all the care and precautions described above. It was at once fixed by flooding with the fixative, and was then treated in the same manner as the normal control specimens.

Microscopic comparison of the capillaries in the omenta of these two series at once revealed striking differences in appearance. The capillaries and the smaller arterioles and venules of the manipulated omenta were distinctly dilated, tortuous, and engorged with blood; furthermore, there was an actual increase in the number of visible capillaries in the handled omenta. It is clear from a study of these slides that many of the fat cells within the fat streaks are bounded by capillaries, that at any given time, under normal conditions, only a relatively small number of these capillaries are open channels for the blood stream, many lying collapsed and closed, and that handling the omentum opens up the collapsed capillaries either by causing a loss of tone through paralysis or by stimulating them to dilate.

The opening up of occult capillaries is seen with especial distinctness along the arterioles and venules which run through the connective tissue between the fat streaks. Clumps of mononuclear cells lie along the course of many of these vessels and form cellular sheaths about them. In the normal omenta these cellular areas are apparently avascular. It is only occasionally that a delicate capillary loop can be seen outlined between the cells. But in the handled omenta these cell clumps are invariably traversed by rich, engorged capillary plexuses, so that the arteriole and venule are bordered by a network of capillaries which can easily be traced to their origins from the arteriole and venule. This appearance is so striking that the handled omenta can be quickly distinguished from the normal omenta by an inspection of these areas.

These observations are entirely in accord with Krogh's<sup>6</sup> demonstration that normally, at any given time, only a relatively small

<sup>6</sup> Krogh, A., *J. Physiol.*, 1918-19, lii, 457.

portion of the entire capillary bed is an open channel for the blood stream, many capillaries remaining collapsed and microscopically invisible; and that these collapsed capillaries open up under conditions which excite their dilatation.

It was evident from a study of these slides that the capillaries were dilated by the manipulation necessary for the placing of the omentum upon the microscope stage; and this fact made it clear that no trustworthy conclusions concerning the effect of histamine upon capillaries could be drawn from such microscopic observations *in vivo*.

*Local Application of Histamine.*—Since the method of fixation of the omentum had yielded such distinctive results, it was utilized in an attempt to study the reaction of the capillaries under the influence of histamine. Two series of experiments were carried out. The animals in the first series were treated as follows: After 20 minutes anesthesia the abdomen was opened along the midline and 10 cc. of normal saline solution warmed to 37°C. were immediately poured over the omentum. The edges of the incision were quickly drawn together and held closed. The omentum was not handled in any way. 1½ minutes after the saline solution had been applied, the edges of the incision were retracted and the omentum was fixed by flooding with the fixing solution. It was quickly removed and permanent stained preparations were made as in the previous experiments.

Microscopic study of these omenta showed that the capillaries, arterioles, and venules presented an appearance in no way distinguishable from those of the previous experiments in which the omenta were immediately fixed upon opening the abdomens. It was apparent, then, that the application of saline solution had not affected the capillaries in any way, and the blood vessels in omenta of this series were considered to exhibit the normal vascular appearance (Figs. 1 and 2).

In the second series of experiments the technique was the same as in the first, except in one respect. The period of anesthetization was the same, the solutions, the temperatures, and the length of time between the saline applications and fixation were identical, but in these experiments histamine was dissolved in the saline solutions, so that the histamine proportion in various experiments ranged from 1:1,000 to 1:20,000. The omenta from these two series of experi-

ments thus afforded for study capillaries which had been subjected to local applications of histamine, and controls in which, under the same conditions, normal saline solution had been applied locally instead of histamine. Microscopic study of these preparations showed that the capillaries of the omenta which had been brought in contact with histamine were very markedly dilated, tortuous, and engorged with blood (Fig. 3) in comparison with the capillaries in the normal controls. There was also seen a distinct increase in the number of visible capillaries. The dilatation was not confined to the capillaries but included the smallest arterioles and venules at the periphery of the capillary bed. The local application of histamine clearly brings about a marked dilatation of both the visible and occult capillaries. That this effect is local was demonstrated by blood pressure tracings taken during the experiments. The pressure was maintained, with the usual slight fluctuations, at a constant normal level, indicating that during the period of contact of the capillaries with histamine not enough of the base had been absorbed to exert the slightest depressor action upon the circulation. When strong histamine solutions were used in the local applications a fall of blood pressure occurred, indicating sufficient absorption to produce the characteristic depressor effect, but the series examined for the purely local effects of the base comprised the omenta only from animals which had exhibited no blood pressure disturbance during the application.

*Histamine Shock.*—In another series of experiments the animals were thrown into shock by intravenous injections of histamine dissolved in 2 cc. of normal saline solution, 2 mg. of the base being used per kilo of body weight. When the blood pressure had fallen to the shock level the abdomen was opened and the omentum immediately fixed as described above. As controls, the omenta of animals which had been given an intravenous injection of 2 cc. of normal saline solution instead of histamine were fixed, the time between the injection and the fixation of the omentum being the same in the two series. The omenta of these controls exhibited a vascular picture differing in no way from that of the normal controls previously described. The capillaries and smaller arterioles and venules of the shocked animals, on the other hand, were definitely dilated and

engorged with blood; and here again it was clear that many occult capillaries had been opened up (Figs. 4 and 5). A study of these slides made it evident that a widespread peripheral vascular dilatation of such a degree could easily accommodate an amount of blood large enough seriously to impair the circulation.

These experiments demonstrated that histamine exerts a local dilating effect upon capillaries and that during histamine shock the blood stream moves sluggishly through a dilated, engorged capillary bed. It was now of importance to determine the relation of the capillary dilatation to the fall in blood pressure which occurs during shock.

*Relation of Capillary Dilatation to Blood Pressure Fall in Shock.*—Experiments were made in which the abdomens were opened and the omenta fixed at various intervals following the injection of histamine. It was found from a study of these preparations that the capillary dilatation had begun in the omentum taken 15 seconds after the injection, before the blood pressure had fallen to the shock level. The progression of the dilatation to a maximum could be followed by a study of the omenta taken at intervals of 15 seconds. No recovery from this peripheral vascular dilatation has been encountered; omenta fixed as late as an hour after the onset of shock have shown the persistence of the dilatation, which apparently must be looked upon as a paralytic loss of capillary tone. The dilatation was uninfluenced by the brief secondary rise in blood pressure which characteristically follows the injection of histamine, and which Dale and Laidlaw have attributed to a constriction of the pulmonary arteries. Omenta fixed at the summit of this rise exhibit the vascular dilatation. This fact makes it clear that the capillary dilatation and engorgement occurring during shock are not a mere result of low blood pressure; the fall of blood pressure is more properly to be attributed to the peripheral vascular relaxation.

There has been ample opportunity during this study to confirm the observations of Dale and Laidlaw<sup>2</sup> regarding the excellent functional condition of the heart in histamine shock. The fall in blood pressure cannot be attributed to cardiac failure. It has frequently been observed, in the present experiments, that during unrecoverable shock the heart efficiently discharges relatively large amounts of

saline solution injected into the jugular vein. In a number of instances repeated intravenous injections of adrenalin have been administered to animals in deep shock, and the heart has always worked with remarkable efficiency during the height of the blood pressure rise. Death during shock has always resulted from respiratory rather than cardiac failure.

The inadequate venous return to the heart has also been observed, and an inspection of the great veins makes it clear that the blood is not accumulating in that portion of the vascular system. The present experiments demonstrate that histamine exerts a local dilator effect upon capillaries and that during histamine shock there are marked widening and engorgement of the capillary bed. The capillary dilatation during shock is accompanied by a fall in blood pressure, but persists independently of subsequent blood pressure variations. These facts are entirely in accord with the view of Dale and Laidlaw that the fall of blood pressure during histamine shock is due to a general loss of capillary tone produced by the poisonous local action of the base.

#### CONCLUSIONS.

1. Histamine exerts a local dilator effect upon capillaries and upon the smallest arterioles and venules which border the capillary system. There occurs also an opening up of large numbers of capillaries of which no trace can be seen before the application of histamine.

2. When injected intravenously in amounts sufficient to produce shock, histamine causes a quickly progressive dilatation of both the visible and the occult capillaries and of their immediately adjacent arterioles and venules, all of which become engorged with blood that moves through them in a strikingly sluggish manner. The circulatory failure which characterizes histamine shock results from the dilatation of the peripheral vascular bed.

## EXPLANATION OF PLATES.

The five photomicrographs were taken with the same magnification.

## PLATE 13.

FIG. 1. Fat streak in the omentum. Local application of normal saline solution.

## PLATE 14.

FIG. 2. Arteriole and venule in the normal omentum showing the apparently avascular cell sheath.

## PLATE 15.

FIG. 3. Fat streak in the omentum. Local application of histamine. Blood pressure normal.

## PLATE 16.

FIG. 4. Fat streak in the omentum during histamine shock.

## PLATE 17.

FIG. 5. Arteriole and venule in the omentum during histamine shock, showing capillary network throughout the cell sheath.

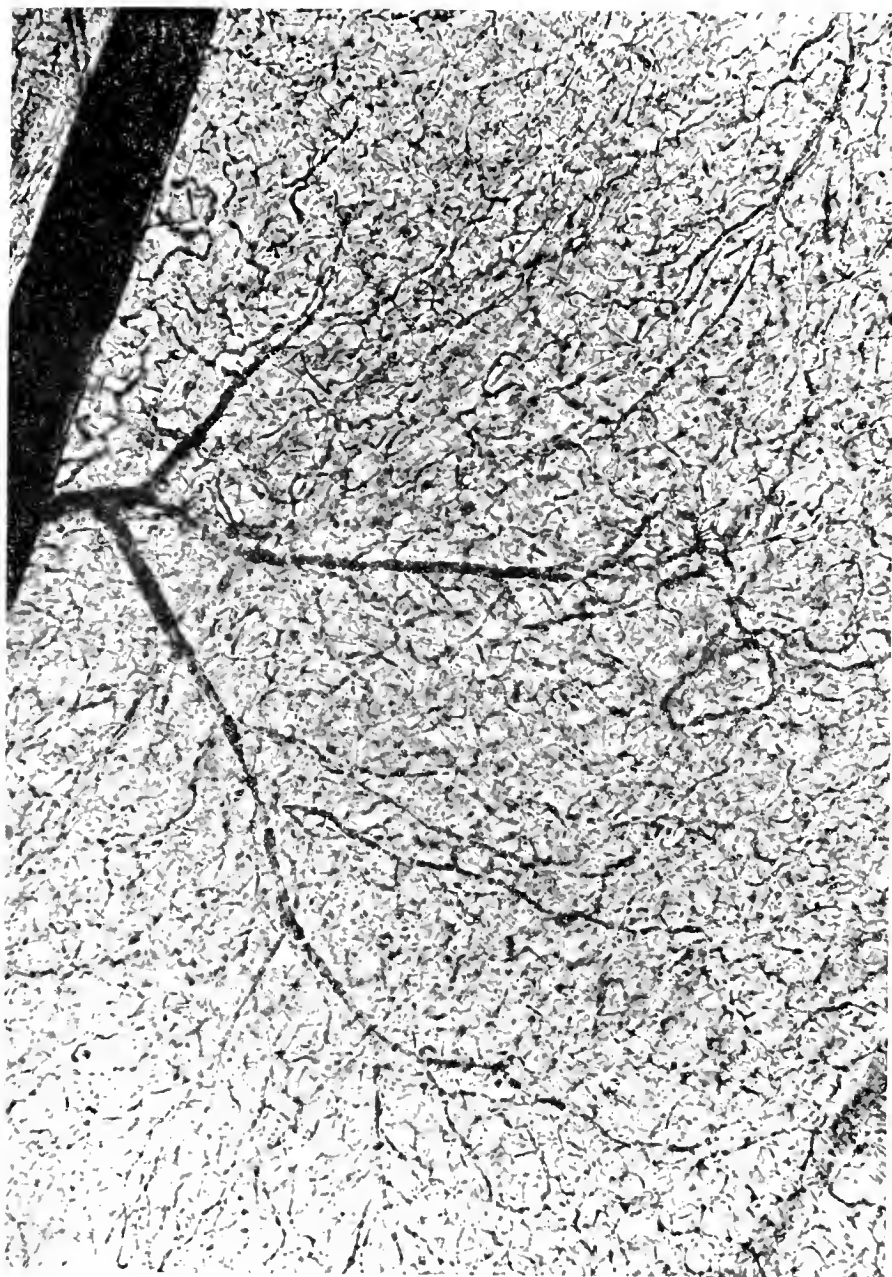


FIG. 1.

(Rich: Capillaries in histamine shock.)





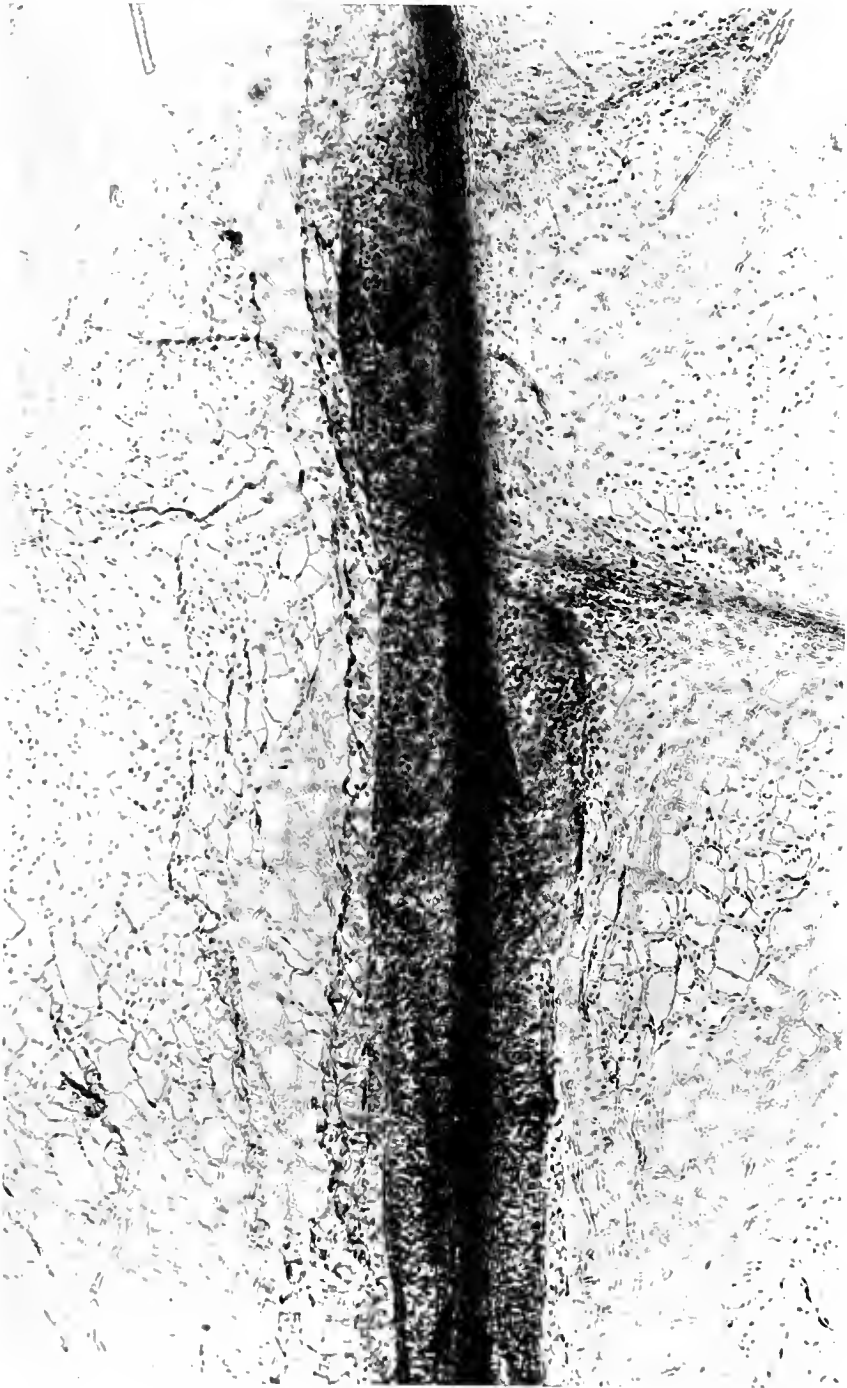


FIG. 2.

(Rich: Capillaries in histamine shock.)



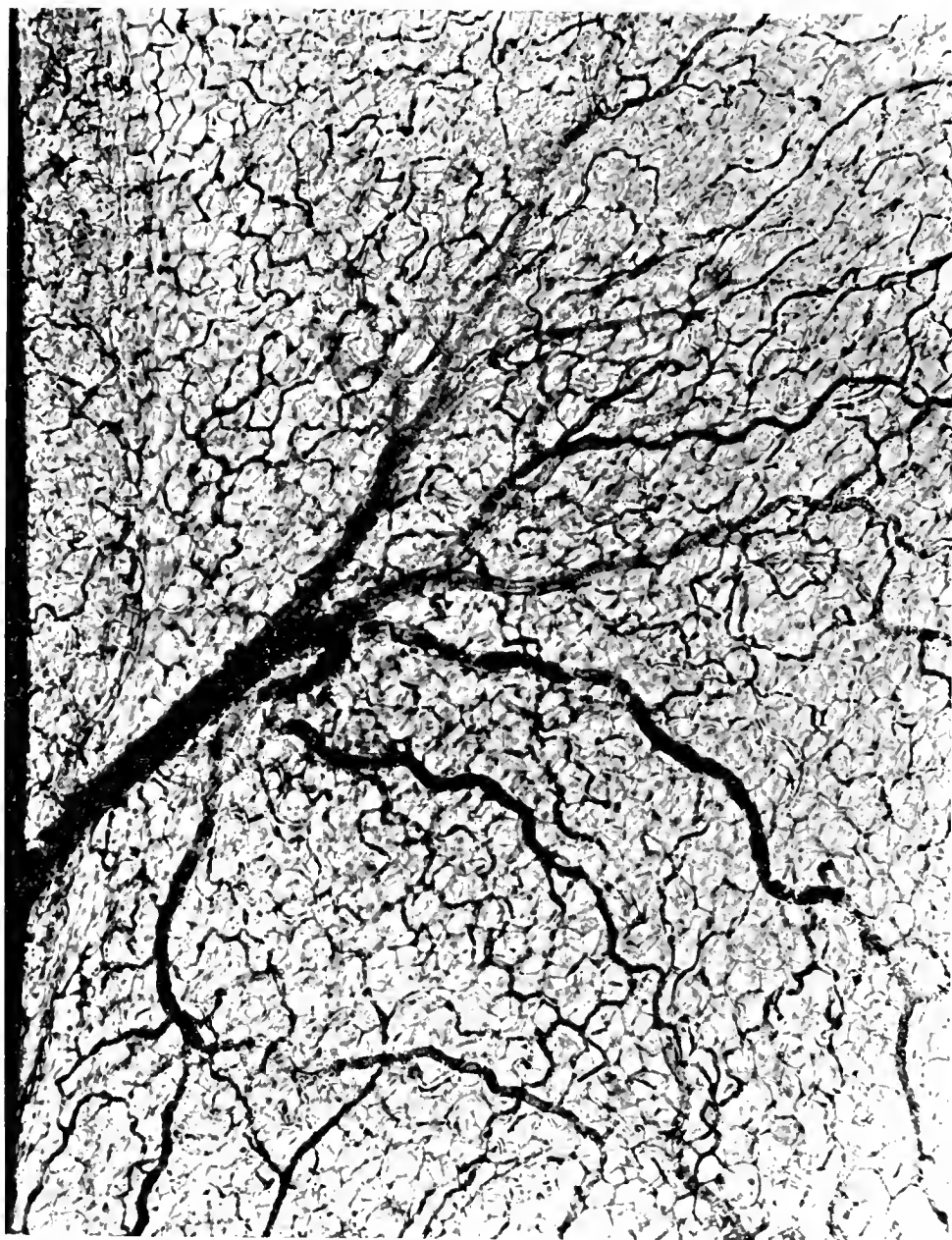


FIG. 3.

(Rich: Capillaries in histamine shock.)



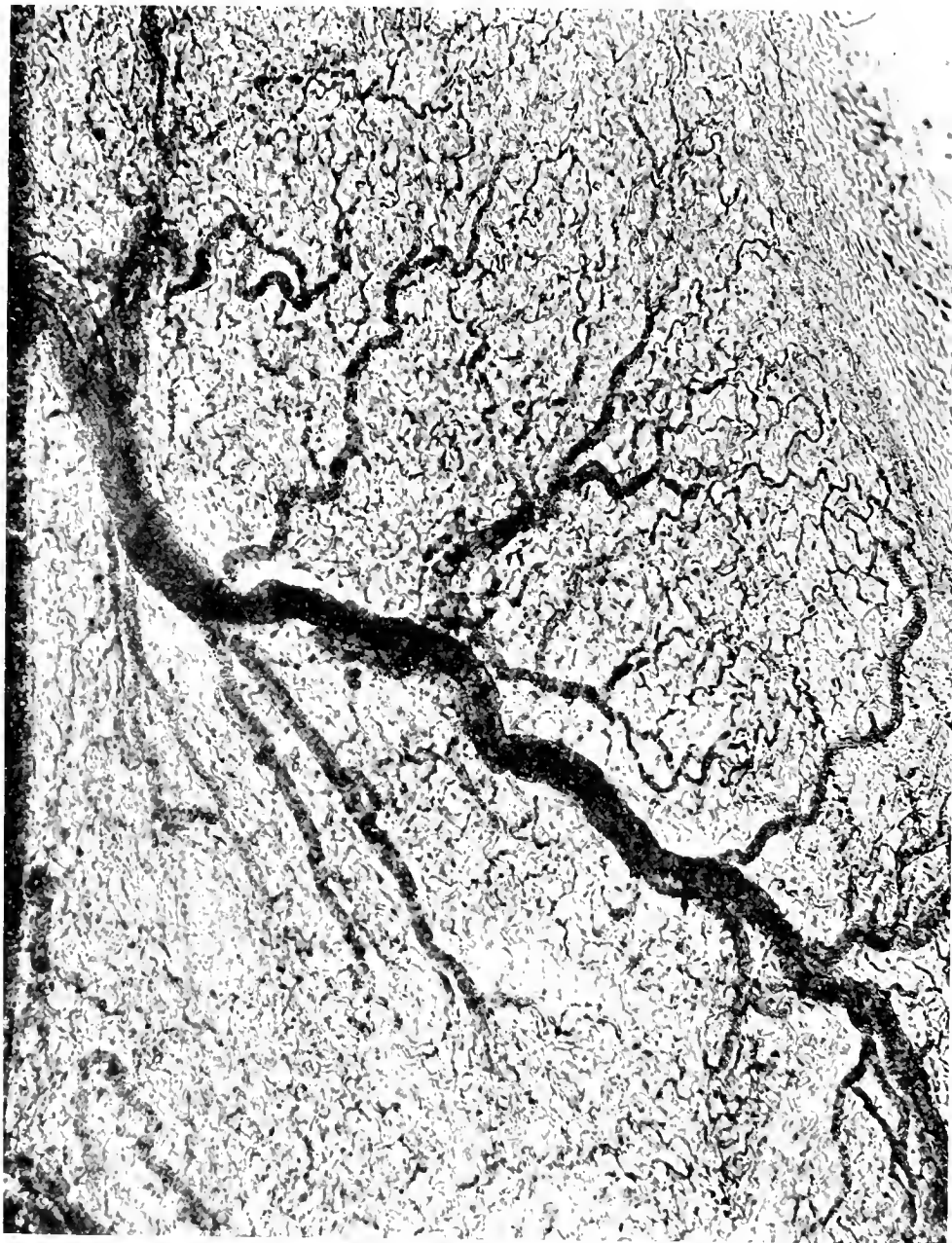


FIG. 4.

(Rich: Capillaries in histamine shock.)



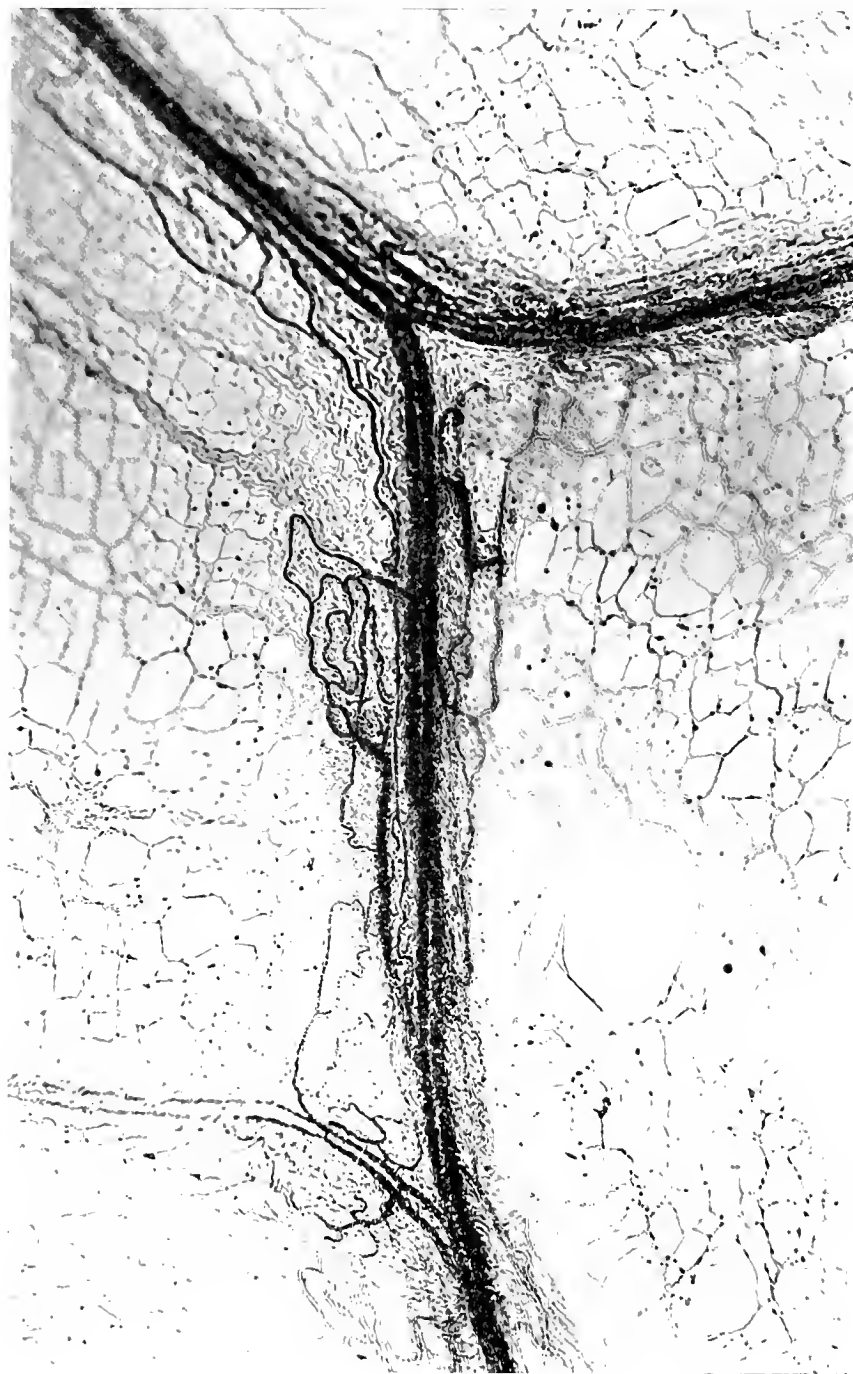


FIG. 5.

(Rich: Capillaries in histamine shock.)





## STUDIES ON X-RAY EFFECTS.

### VI. EFFECT OF THE CELLULAR REACTION INDUCED BY X-RAYS ON CANCER GRAFTS.\*

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PLATES 18 TO 20.

(Received for publication, October 19, 1920.)

The theory that cancer tissue in general is more susceptible to injury by x-rays than is normal tissue has been the subject of extensive investigation.<sup>1</sup> As far as we have been able to determine from the literature there have been no conclusive proofs brought forward that this theory is correct. Cancer cells can undoubtedly be killed by x-rays, but judging from our experiments the amount of x-rays which can safely be given to man, without causing burns and other deleterious effects, is not sufficient to kill the cancer cell *in vitro*.<sup>2</sup>

In general it may be said that the x-rays have given almost uniformly beneficial results in the treatment of human cancer in only one type of malignant disease; namely, skin cancer, particularly of the basal cell epithelioma type. Many explanations have been brought forward to account for the fact that these growths are so easily affected, while other cancers, lying just beneath the skin and therefore almost as accessible to the rays, yield less uniform results. The belief among x-ray workers is that the difference depends on dosage, and their tendency has been to endeavor to increase the amount of x-rays delivered to the diseased area. Even very large doses of x-rays have failed to give uniformly good results in any but the super-

\* This investigation was carried out by means of funds from the Rutherford Donation.

<sup>1</sup> Colwell, H. A., and Russ, S., Radium, x-rays and the living cell, London, 1915, 270.

<sup>2</sup> Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

facial cancers, although sometimes there is a slowing down of the progress or even a cessation of growth for a period; rarely has actual retrogression taken place. In the vast majority of instances the treatment may be said to have had no effect, and even in the few cases in which there is improvement the benefit is only temporary.

Aside from skin cancer the only other tumors greatly affected by x-rays are the sarcomata arising from the testicle, and certain lymphoid tumors. It is of interest to note that the tissues from which these tumors arise are the most sensitive of the normal tissues to x-rays. Here again it is doubtful whether the malignant tissue is any more sensitive than the normal tissue from which it arose. Some of those who question whether the cancer cell is more susceptible than normal tissue to x-rays have advanced theories to explain their results. The two which have received most attention are (a) the idea that the dividing cell has an increased susceptibility to x-rays, and (b) the theory that the effect obtained from x-ray therapy arises from the induced obliteration of the blood vessels which diminishes the nutrition delivered to the new growth. If the first of these explanations is correct, we should expect that the best method of treating a skin cancer would be by very frequent small doses of x-rays so as to destroy the cells as they reach the dividing stage. Experience has shown that this method does not give the desired result. If the obliteration of the blood vessels be the cause of the occasional retrogression, we should expect a more uniform result than is at present obtained, for blood vessel changes are quite constant.

In the past, investigators in this field have attempted to establish the efficacy of x-ray therapy by exposing tumors *in situ*. The results viewed from the experimental side have one fallacy; namely, that they do not take into account the effect of x-rays on the animal in general and the local tissue reaction induced by this agent. Other experimenters have exposed tumor grafts *in vitro* to x-rays and then inoculated them into animals. In most instances when destruction of the cancer grafts resulted, the doses used were not comparable to the amount of x-rays that can safely be given to a living animal. When no definite destructive action on the cancer grafts was noted with amounts of x-rays comparable to the dose which can safely be given to man, the view was put forward that as only one dose of

x-rays was administered in this case, while in the usual treatment of man repeated exposures are given, no conclusions can be drawn from the failure. We have recently reported a series of experiments in which the attempt was made to treat a transplantable mouse cancer *in vitro*, as nearly as possible in the same manner as that employed in the treatment of human cancers, with the exception that the dosage was magnified.<sup>2</sup> The cancer growths were removed at intervals of from 5 to 6 weeks, exposed to x-rays, and replanted in a new series of animals. The outcome of the experiment was at first to reduce slightly and transitorily the growth energy of the tumor, while the later treatments were without effect either on the number of takes or the rate of growth of the tumor.

Hence it may be assumed that considerable doubt still exists that x-rays in a dose suitable for a living animal, *i.e.* an amount which will not produce a burn, will exert a very great destructive action on the cancer cell. The question arises why uniformly good results should follow the treatment of skin cancer and almost as uniformly poor results be obtained in the treatment of cancers in only slightly deeper tissues. The problem involved is the immediate basis of the experiments to be described in this paper.

The studies carried out by workers in this laboratory, extending over several years, have emphasized the close relation existing between the lymphocytes and resistance or susceptibility to cancer growths.<sup>3</sup> They have also shown that the lymphocyte is greatly affected by x-rays, since it is possible either to stimulate by small doses the production of these cells or by larger ones practically to destroy all the lymphoid tissues of the body.<sup>4</sup> In looking for an explanation of the results of the treatment of human cancer with x-rays, in the light of the findings described above, we have noted two interesting observations in the literature—first, that in treating cancer of the skin the

<sup>3</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25, 31. Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

<sup>4</sup> Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

method found to be best is the one in which a dose sufficient to produce a so called x-ray erythema is given, the dose not being repeated until this erythema has subsided;<sup>5</sup> and second, that the so called x-ray erythema, when studied histologically, consists, besides the dilation of the blood vessels, etc., of a lymphoid infiltration of the skin layers,<sup>1</sup> which, however, does not extend to the subcutaneous or deeper tissues. Hence, it seemed not impossible that this important difference might account for the discordant results of the treatment of cancers of the skin and of the deeper tissues. The following experiments were planned to test this point.

#### EXPERIMENTAL.

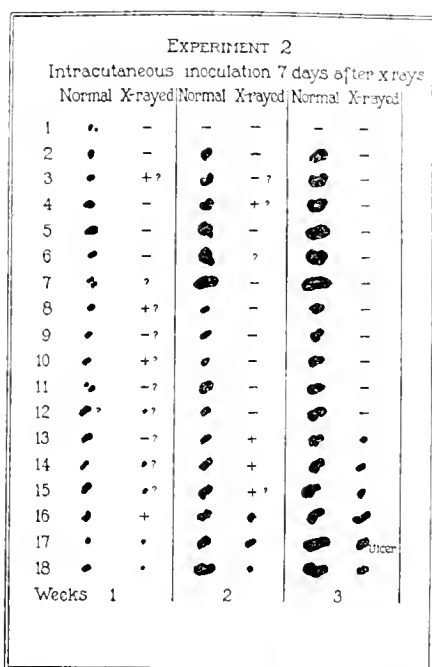
*Intracutaneous Inoculation 7 Days after Exposure to X-Rays.*—The region extending from the upper abdomen down to and including both groins was shaved carefully in healthy young mice. These animals were secured on a small board and the entire body was covered with sheet lead. An opening 15 by 20 mm. was cut in the lead so as to expose a region in the left groin extending to the midline, and this area was then exposed to x-rays in the following dose: 3 inch spark-gap, 10 milliamperes, 6 inch distance from target, and  $2\frac{1}{2}$  minutes exposure. About 7 days later the skin showed a mild erythema with some scaliness. At this period small grafts of young healthy tumor from the Bashford adenocarcinoma, No. 63 series, were inoculated intracutaneously in the center of the x-rayed area and also in the corresponding position of the protected right groin. On account of the thinness of the skin, considerable caution was necessary to avoid thrusting the grafts through into the subcutaneous tissue, but occasionally this accidentally occurred. Weekly observations and measurements were made. The results of the individual experiments are shown in Table I and Text-figs. 1 and 2.

Thus it appears that there is a decided difference in the number of takes from cancer grafts inoculated into the skin of an area previously exposed to an erythema dose of x-rays, as compared to the

<sup>5</sup> Knox, R., Radiography, x-ray therapeutics, and radium therapy, New York, 1916.

TABLE I.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		<i>per cent</i>	<i>per cent</i>
1	10	40.0	90.0
2	18	33.3	94.4
3	20	40.0	100.0
4	9	44.5	100.0



TEXT-FIG. 1. The growth of cancer grafts inoculated intracutaneously in an area 7 days after an x-ray exposure, compared to the growth of similarly inoculated grafts in an untreated area in the same animals.

number when the grafts are inoculated in the same manner into the same animal, but in an area protected from x-rays (Fig. 1). The next question to arise was whether the difference can be explained by the mechanical interference with the blood supply in the x-rayed area, from the induced changes in the blood vessels. To determine

this point a number of animals from this series were killed with ether and skinned. The skin was held before a light, by which means a clear definition of the vessels is secured. The vessels in all instances were found to be distended and numerous around the grafts in both areas, and whenever a difference was noted it was in favor of the x-rayed side. Moreover, another series of animals was injected with

EXPERIMENT 3									
Intracutaneous inoculation 7 days after x-rays									
Normal		X-rayed		Normal		X-rayed		Normal	
1		+	?		-	?		-	
2		-			-			-	
3	+	+	?		+	?		-	
4		+	?		-	?		-	
5		+	?		-			-	
6		-	?		-	?		-	
7		+	?		+			-	
8		+	?		-			-	
9	-	-	?		-			-	
10		-	?		-	?		-	
11		-	?		-			-	
12		+	?		+	?		-	
13		-	?		+	?		-	
14		+	?		+	?		-	Ulcer
15		-	?		-			-	
16		+	?		+			-	
17		+	?		-	?		-	
18		+	?		+			-	
19		+	?		-			-	
20		-	?		-			-	Ulcer
Weeks 1		2		3					

TEXT-FIG. 2. A repetition of the experiment shown in Text-fig. 1.

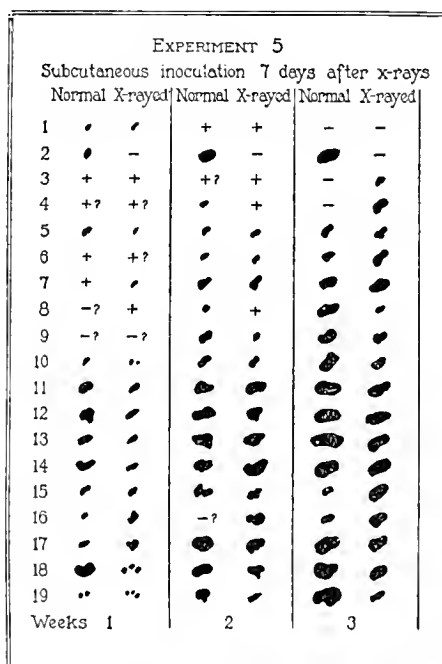
India ink into the heart, whereby a satisfactory injection of the superficial vessels was obtained. No essential difference in the number of patent vessels on the two sides was detected.

We return now to the second possibility. If the induced cellular reaction in the skin accounts for results obtained in the treatment of human cancer, we should expect no difference in the number of takes and the rate of growth of the cancer grafts in mice inoculated under the skin rather than into the skin of an x-rayed area. The following experiments were planned to test this point.

*Subcutaneous Inoculation 7 Days after Exposure to X-Rays.*—A series of mice was shaved in the same manner as those described in the preceding experiments and an area on the left side of the abdomen was exposed to x-rays in the same dose, the rest of the body being completely protected. A week later a cancer graft was inoculated into the x-rayed area, but just under the skin in the loose connective tissue. Another graft was inoculated in the same manner on the opposite side of each animal, in the area protected from x-rays. The results are shown in Table II and Text-fig. 3.

TABLE II.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		<i>per cent</i>	<i>per cent</i>
5	19	89.5	84.2
6	9	88.9	88.9



TEXT-FIG. 3. A graphic representation of the results of subcutaneous inoculations of cancer grafts into x-rayed areas as compared with the subcutaneous inoculations into untreated areas.

From the foregoing data it will be seen that the cancer graft inoculated into an x-rayed area, but under the skin instead of into the skin, grows equally as well as does a graft in a protected area inoculated in the same manner. It would seem, therefore, that whatever change is induced by x-rays which renders a tissue unsuitable for the growth of cancer is confined to the skin and does not extend even to the loose connective tissue just below it. The histological changes induced by the x-rays were next studied.

*Histological Examination of Tissues of Animals Inoculated Intracutaneously after Exposure to X-Rays.*—A series of mice was shaved and treated with x-rays in the same manner as in the foregoing experiments. Some of these were killed on the 3rd day and others on the 7th day after treatment. The remaining mice were inoculated intracutaneously with a graft of Bashford Tumor No. 63 in the center of the x-rayed area and in a corresponding location in the protected groin. These last animals were killed off in groups for histological study 48 hours, 4 days, and 7 days after inoculation.

The histological examination showed that 3 days after exposure to the x-rays the skin was practically normal, while 7 days after treatment a marked accumulation of lymphoid varieties of cells was present, particularly in the stratum papillare of the corium in the x-rayed area (Fig. 2), whereas the untreated area remained entirely normal (Fig. 3).

The tumor grafts in the specimens taken after inoculation were found to lie in the tela subcutanea, just beneath the stratum reticulare. In the instances in which the graft was not completely destroyed in the x-rayed area, there was a marked lymphoid reaction about it (Fig. 4), in striking contrast to the practical absence of reaction around the graft in the normal area (Fig. 5). After the destruction of the tumor graft was complete, the lymphoid infiltration in the x-rayed area of the skin was less marked.

The next experiments were made to ascertain the period of maximum effect of the changes induced in the skin by x-rays.

*Exposure to X-Rays 20 Hours after Intracutaneous Inoculation.*—A series of mice was shaved over the abdomen and inoculated intracutaneously in both groins as in the previous experiments. 20 hours after the tumor inoculation the graft in the left groin with an area



around it was exposed to x-rays in a dose governed by the following factors: spark-gap 3 inches, milliamperes 10, distance from target 6 inches, and time  $2\frac{1}{2}$  minutes, a dose previously determined to be insufficient to destroy the cancer cell. The animals were examined weekly and measurements made of the tumors with the results shown in Table III and Text-fig. 4.

TABLE III.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		<i>per cent</i>	<i>per cent</i>
7	7	14.4	83.4
8	11	10.0	63.7
9	20	15.0	85.0
10	18	50.0	83.4

As a control to the above observations a 3 weeks old cancer from the same series as that used for the preceding experiments was cut up into 48 small bits. These were then divided into two lots, each containing twenty-four pieces, and one lot was exposed to a dose of x-rays in the same amount as that given in the previous experiment to the area of skin in the groin. The x-rayed particles of tumor were then inoculated intracutaneously in the left groin of twenty-four mice and at the same time one of the untreated tumor particles was inoculated intracutaneously into the right groin of each mouse. At the end of 3 weeks eighteen of the twenty-four untreated grafts had produced tumors and sixteen of the twenty-four x-rayed grafts had grown. There was no appreciable difference either in the time of appearance or the rate of growth of the tumors in the two sides.

*Histological Study.*—Ten normal white mice were inoculated intracutaneously in both right and left groins with the strain of transplantable cancer used before. 24 hours later x-rays, governed by the same combination of factors as before, were given on the left groin over the skin area in which the cancer graft had been implanted. The right groin was left untreated for comparison. The mice were killed in groups of two, immediately, after 24 hours, 48 hours, 4 days, and 7 days after x-ray exposure.

EXPERIMENT 9												
Intracutaneous inoculation												
X-rayed 20 hours after inoculation												
	Normal		X-rayed	Normal		X-rayed	Normal		X-rayed	Normal		X-rayed
1	-?	+	?	-	-	-	-	-	-	-	-	-
2	-?	-	-	-	-	-	-	-	-	-	-	-
3	•?	-?	-	-	-	-	-	-	-	-	-	-
4	•	-?	•	-	-	•	-	•	-	•	-	-
5	•	-	•	-	-	•	-	•	-	•	-	-
6	•	+	•	-	-	•	-	•	-	•	-	-
7	•	+	•	-	-	•	-	•	-	•	-	-
8	•	-?	•	-	-	•	-	•	-	•	-	-
9	•	+	•	-?	-	•	-	•	-	•	-	-
10	•	+	•	-	-	•	-	•	-	•	-	-
11	•	+	•	-	-	•	-	•	-	•	-	-
12	•	-	•	-	-	•	-	•	-	•	-	-
13	•	-?	•	-	-	•	-	•	-	•	-	-
14	•	+	•	-	-	•	-	•	-	•	-	-
15	•	•?	•	-	-	•	-	•	-	•	-	Died
16	•	+	•	-	-	•	-	•	-	•	-	-
17	•	-	•	-	-	•	-	•	-	•	-	-
18	•	+	•	•?	-	•	-	•	-	•	-	-
19	•	+	•	•	-	•	-	•	-	•	-	-
20	•	•	•	•	-	•	-	•	-	•	-	-
Weeks	1		2		3							

TEXT-FIG. 4. The result of an experiment in which a cancer was inoculated intracutaneously into each groin of twenty mice and 20 hours later a dose of x-rays was given over the left groin so as to include the cancer graft and the surrounding tissue.

No detectable histological difference was found in the skin of the two sides of animals killed during the first three periods. The moderate cell infiltration about the graft in the treated, as well as the untreated side, consisted chiefly of polymorphonuclear leucocytes.

Beginning with the 4 day period an extensive lymphoid infiltration in the skin, especially about the graft on the treated side, appeared, while the graft on the untreated side was well established and attended by a moderate cell infiltration, in which polymorphonuclear cells were taking the more prominent part.

By the 7th day the tumor graft had disappeared in the treated side, but an intense lymphoid infiltration of the skin was present. In the untreated side a growing tumor was found, accompanied by some cell infiltration, although the adjacent skin showed only slight invasion.

EXPERIMENT 12					
Intracutaneous inoculation 2 hours after x-rays					
	Normal X-rayed	Normal X-rayed	Normal X-rayed	Normal X-rayed	Normal X-rayed
1	• -?	- -	- -	- -	- -
2	+ •	- -	- -	- -	- -
3	•? +?	- -?	- -	- -	- -
4	- +?	+? -?	- -	- -	- -
5	+ +?	- -	- -	- -	- -
6	• -?	• -?	• -?	• -	- -
7	• +	• -?	• -?	• -	- -
8	• -?	• -	• -	• -	- -
9	+? +?	• -	• -	• -	- -
10	• -?	• -?	• -?	• -	- -
11	• •	• +?	• -	• -	- -
12	• •?	• -?	• -?	• -	- -
13	• +?	• -?	• -?	• -	- -
14	• •	• +?	• -	• -	- -
15	• •	• -?	• -?	• -	- -
16	• +?	• -?	• -?	• -	- -
17	• +?	• -?	• -?	• -	- -
18	• •	• +?	• -?	• -	- -
19	• •	• -?	• -?	• -	- -
20	• •	• -?	• -?	• -	- -
21	•? •	• -?	• -?	• -	- -
22	•? •?	•? •	• -?	• -	- -
23	• •	• +?	• -?	• -	- -
24	•? +?	• +	• -?	• -	- -
Weeks	1	2	3		

TEXT-FIG. 5. The growth of cancer grafts inoculated intracutaneously in an area of skin 2 hours after the skin had been exposed to x-ray treatment compared to the fate of similarly inoculated grafts in an untreated area in the same animals.

While this dose of x-rays has been shown to be incapable of destroying tumor cells *in vitro*, the objection to this result as a confirmation of our first experiments is obvious, as the cancer cells in the latter experiments were exposed to the direct action of the x-rays. The following experiments were planned with the idea of avoiding this

objection and yet availing ourselves of the full time of the effect produced by the x-rays in the skin.

*Intracutaneous Inoculation 2 Hours after Exposure to X-Rays.*—Mice were shaved over both groins and then given the same dose of x-rays over the left groin as that given in the preceding experiments. 2 hours later intracutaneous inoculations of cancer grafts were made into the x-rayed area of the left groin and in the corresponding locality in the right groin, the latter having received no x-rays. The results 3 weeks after these inoculations are given in Table IV and Text-fig. 5.

TABLE IV.

Experiment No.	No. of animals.	Growth in x-rayed area.	Growth in protected area.
		<i>per cent</i>	<i>per cent</i>
11	18	38.9	88.9
12	24	12.5	75.0

It will be seen from these last two groups of experiments that when the inoculation is made either just before or just after the administration of x-rays, the results are only slightly if any better than when the cancer inoculation is made when the reaction in the skin is at its height.

#### DISCUSSION AND CONCLUSIONS.

We shall not attempt to discuss the complex question of the amount of x-rays required to kill the cancer cell, for this has been dealt with extensively in recent literature.<sup>6</sup> Certain facts stand out which cannot be satisfactorily explained by the direct action theory; namely, that in man skin metastases are often easily influenced by x-rays while the primary growth or even metastases in the subcutaneous tissue are resistant. We have seen such a case in our clinic at the Hospital of The Rockefeller Institute, in which numerous skin metastases disappeared under mild doses of x-rays while metastases in the superficial glands of the neck and axilla showed no retrogression even under large doses. The amount of x-rays delivered to the cancer cells in the latter instances was many times greater than that given to the skin metastases. The experiments reported in this paper

<sup>6</sup> For a review of recent literature see Wood, F. C., and Prime, F., *J. Am. Med. Assn.*, 1920, lxxiv, 308.

offer a plausible explanation of this phenomenon. When the metastases or primary growth is in the skin, the x-rays induce a condition which renders it an unsuitable soil for survival of the tumor but this change does not extend as far beyond the skin layers as the subcutaneous tissue. The x-rays bring about a marked cellular reaction confined to the skin layers. It seems probable, therefore, that the explanation of the x-ray action in rendering an area unsuitable for cancer growth is the local cellular reaction induced in this tissue. Thus this effect is brought into harmony with the observations already published by us on the relation of lymphoid cell reaction and resistance to tumor growth. Certain studies recently published by Ewing<sup>7</sup> on the effect of radium in the treatment of human cancers indicate that a similar mechanism plays a part in the beneficial effects obtained by this mode of treatment.

Another explanation which has been proposed by many observers is that the good effect of x-rays depends on the induced blood vessel changes with a resultant deficiency of nutrition to the tumor cells. Our experiments covering this point showed that at no stage of the erythema or later during the retrogression of the tumor could any evidence of obstruction to the local blood supply be detected. In the light of the previous experiments on the relation of the lymphoid reaction to cancer immunity and the present experiments on the lymphoid cell reaction induced by x-rays and the failure of the growth of tumors in such areas, this central fact must be taken into consideration in accounting for the therapeutic action of x-rays in cancer. In this connection we desire to state that we do not regard the results obtained in the treatment of testicular sarcoma and certain lymphoid tumors as covered by this explanation, for like their parent tissues the cells of these respective tumors are particularly sensitive to the direct action of x-rays.

There remain to be considered the occasional beneficial results obtained with x-rays in metastatic cancer in lymph glands. This question is not a simple one. For example, in regions draining an area affected by cancer, the lymph glands often become more numerous and much larger than normal. Such glands are often regarded

<sup>7</sup> Ewing, J., *J. Am. Med. Assn.*, 1917, lxviii, 1238.

as metastatic, and yet pathologists who have examined the extirpated glands often fail to find cancer cells. These hypertrophic glands would, of course, melt away under x-rays. There is another series of events which may also give a false impression of retrogression of cancer metastases under x-rays. We have seen such an instance in the case of a large gland in the neck of a patient suffering from cancer of the breast. The nodule was exposed to vigorous x-rays and promptly retrogressed to a point where it was just palpable. The small nodule was removed at operation at this period with another involved gland which had not been subjected to the x-rays. In the latter, or untreated gland, there was a small metastasis with a fairly abundant supply of lymphoid tissue, the two making up a fair sized nodule, while in the treated gland the metastasis was found to be made up of healthy tumor cells showing no evidence of deleterious effects from the x-rays. We believe that in this case the apparent retrogression of the nodule was due simply to the destruction of the lymphoid elements of the gland with no effect whatever on the cancer cells. Finally, examples are known of definite retrogression of metastatic nodules in the superficial glands resulting from x-ray treatment to which the above explanations do not apply. Whether these are to be explained by an occasional reaction induced in the deep tissues by x-rays, or whether they represent occasional examples of tumor tissue hypersensitive to x-rays, future studies will have to determine. In view, however, of the doubt surrounding the opinion that cancers in general are more sensitive to x-rays than is normal tissue, we wish to question the advisability of the present tendency to increase greatly the dose of x-rays. We make this point since our previous studies have shown that it is possible in mice to break down the general mechanism of resistance against cancer by overwhelming doses of x-rays.<sup>8</sup>

#### SUMMARY.

Small areas of the skin in the groin of mice were subjected to an erythema dose of x-rays and a week later a cancer graft was inoculated intracutaneously into the area and at the same time a like graft was

<sup>8</sup> Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

inoculated in the same manner in the opposite groin protected from x-rays. The graft in the x-rayed area showed a low percentage of takes, while that in the normal skin gave the usual high percentage. When the graft was introduced into the subcutaneous tissues it grew equally as well in the x-rayed area as in the protected area.

Histological examination shows the skin layers, a few days after x-ray treatment, to be markedly infiltrated with round cells of the lymphoid type. The reaction did not extend deeper than the skin layers. It is suggested that this local lymphoid reaction induced by the x-rays controls the graft made into the skin, while its absence in deeper tissues accounts for the growth of the grafts more deeply implanted.

#### EXPLANATION OF PLATES.

##### PLATE 18.

FIG. 1. The result of an intracutaneous inoculation of cancer grafts in an area previously exposed to x-rays (left side) compared with the result of a similar inoculation in an untreated area (right side).

##### PLATE 19.

FIG. 2. X-rayed area of the skin of a mouse 7 days after the treatment.

FIG. 3. Untreated area of the skin of the same mouse.

##### PLATE 20.

FIG. 4. Cancer graft in an x-rayed area.

FIG. 5. Cancer graft in an untreated area.

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FIG. 1.

(Murphy, Hussey, Nakahara, and Sturm: X-ray effects. VI.)



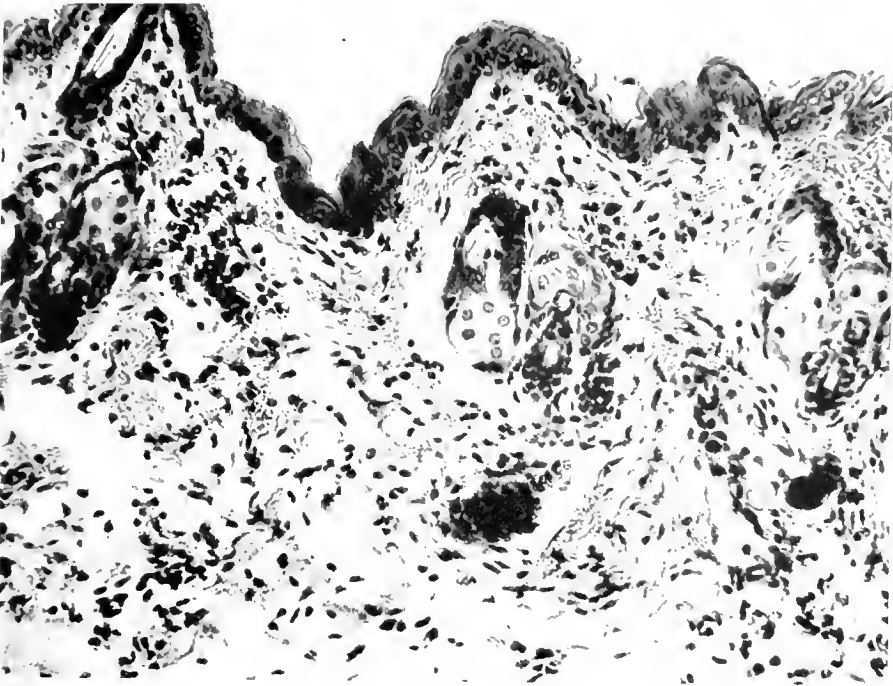


FIG. 2.

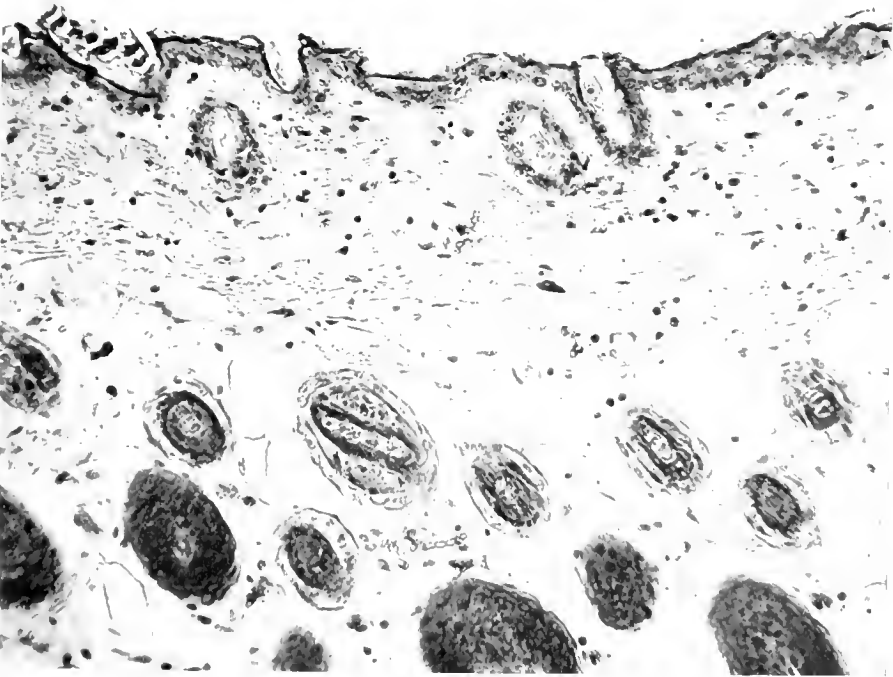


FIG. 3.

(Murphy, Hussey, Nakahara, and Sturm: X-ray effects. VI.)



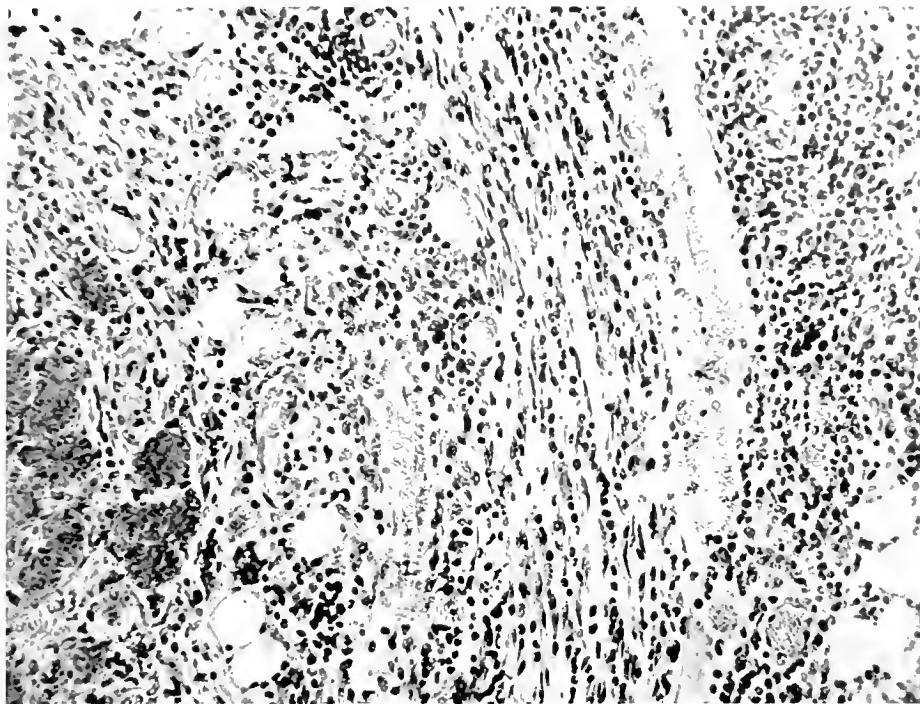


FIG. 4.

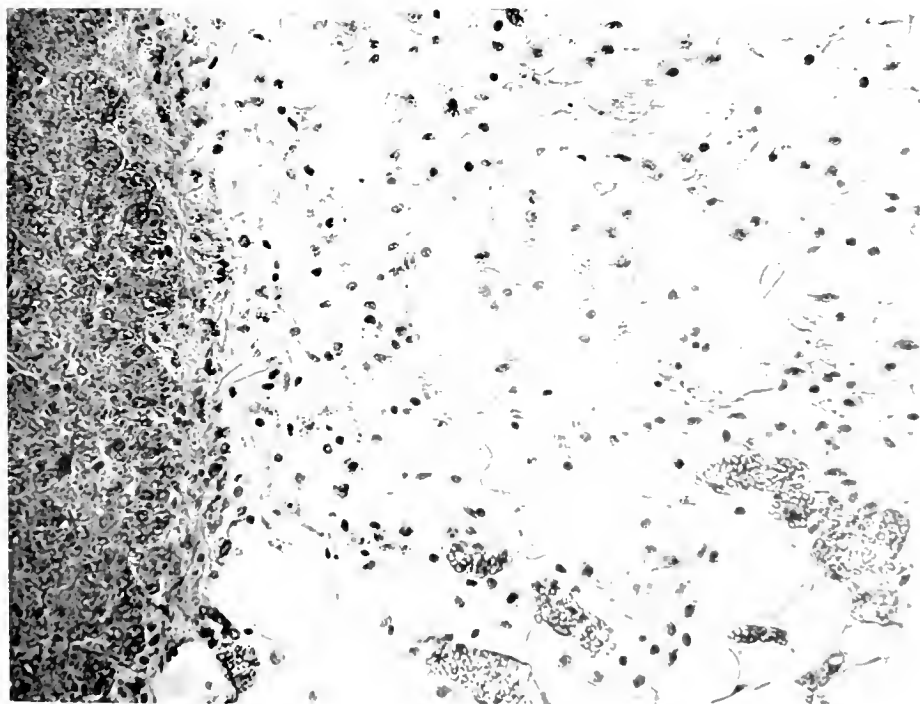


FIG. 5.



# EFFECT OF INDUCED CELLULAR REACTION ON THE FATE OF CANCER GRAFTS.\*

## IV. STUDIES ON LYMPHOID ACTIVITY.

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PLATES 21 TO 23.

(Received for publication, October 15, 1920.)

It has been shown that the induction of a general lymphocytosis is accompanied by a more or less marked immunity to cancer,<sup>1</sup> and that a local reaction of lymphoid cells, induced in the skin by means of x-rays, renders this tissue an unsuitable locality for the growth of cancer.<sup>2</sup> The reaction about a cancer graft inoculated into a mouse previously injected with homologous living tissue has a striking likeness to a local anaphylactic reaction, and is followed by a more or less complete destruction of the tumor graft. Yet in spite of this constant association of immunity and the cellular reaction, cancer investigators have been inclined to look for other explanations of the immunity phenomena.<sup>3</sup> If this cellular reaction is an important factor and appears to explain more or less the immunity phenomena, it should be possible to bring about a local immunity to cancer, by inducing around a graft a reaction similar to that which occurs in a generally immune animal. As the local anaphylactic reaction has

\* This investigation was carried out by means of funds from the Rutherford Donation.

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 25, 31. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

<sup>2</sup> Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

<sup>3</sup> For a review of the literature see Woglom, W. H., *Studies in cancer and allied subjects*. The study of experimental cancer. A review, New York, 1913.

similarities with the local effect observed about a graft in cancer-immune animals, we have tested out the influence of the former reaction on cancer grafts.

### *Foreign Protein Reaction in Mice.*

The material most generally used to produce homologous tissue immunity in mice is defibrinated mouse blood; the amount necessary to induce a satisfactory immunity is about 0.2 cc. In order to parallel closely this procedure the same amount of defibrinated rat blood has been used as the foreign protein in the following experiments.

0.2 cc. of defibrinated rat blood was injected into the loose connective tissues of the backs of mice, the first series of which was killed 24 hours later and the tissues prepared for and subjected to histological examination. A considerable degree of lymphoid infiltration in the region of the injected blood (Fig. 1) was present, similar to that which occurs about an injection of defibrinated mouse blood.<sup>4</sup>

10 days later another series of the sensitized mice was given a second small injection of rat blood into the groin, and after another 24 hours the animals were killed and the groin tissue was prepared for histological examination. This tissue showed a marked infiltration of lymphocytes about the injected blood, a reaction far more extensive than that occurring after a single injection and equal to that found about a cancer graft in an immune animal (Fig. 2).

Hence it is readily possible to induce a reaction to foreign protein similar to that accompanying the immunity reaction to cancer. The following experiments were planned to test the effect of this reaction on cancer grafts.

*Experiment 1.*—A large normal rat was bled from the heart under aseptic precautions and the blood was defibrinated. Of sixteen normal mice from the same strain and of about the same age, eight were injected subcutaneously with 0.2 cc. of the rat blood. 10 days later a 2½ weeks old Bashford adenocarcinoma was removed from a mouse and cut into pieces of approximately equal size. The fragments were placed in a container and thoroughly mixed with a quantity of freshly defibrinated rat blood. The tumor particles were then loaded into a trocar, care being taken to include a drop of the blood with each graft, and inoculated into eight mice formerly injected with rat blood and, in the same manner, into

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<sup>4</sup> Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.



eight normal control mice. Measurements of the grafts were made at weekly intervals. At the end of 3 weeks, of the eight animals which had been sensitized with rat blood and afterwards inoculated with a mixture of rat blood and the cancer, three only showed tumors, while all the control mice inoculated with a mixture of rat blood and the cancer showed tumors.

*Experiment 2.*—The preceding experiment was repeated with ten mice in the sensitized series and ten in the control. Among the former four tumors developed, or 40 per cent of takes, while among the latter nine tumors developed, or 90 per cent of takes.

*Experiment 3.*—Twenty mice were given an injection of 0.2 cc. of defibrinated rat blood subcutaneously. 10 days later ten of these were inoculated with a mixture of rat blood and mouse cancer, and the other ten with mouse cancer alone. A control series of ten non-sensitized mice was also inoculated with a mixture of cancer and rat blood. The mice sensitized and inoculated with a mixture of rat blood and cancer showed four tumors, or 40 per cent of takes, the mice sensitized and injected with cancer grafts alone showed ten tumors, or 100 per cent of takes, and the control, non-sensitized mice given cancer plus blood showed nine tumors, or 90 per cent of takes.

*Experiment 4.*—This experiment was a repetition of Experiment 3, with ten mice in each series. The mice sensitized with rat blood and then inoculated with a mixture of rat blood and mouse tumor showed five tumors, a susceptibility of 50 per cent, the mice sensitized with rat blood and inoculated with tumor alone showed ten tumors, a susceptibility of 100 per cent, and the normal mice inoculated with a mixture of rat blood and mouse tumor showed ten tumors, a susceptibility of 100 per cent.

*Experiment 5.*—The experiment was again repeated with ten mice in each series. The mice sensitized with rat blood and then inoculated with a mixture of rat blood and mouse cancer showed a susceptibility of 60 per cent, while the animals sensitized and inoculated with cancer material alone were 100 per cent susceptible, as was also the normal control series inoculated with a mixture of rat blood and cancer.

*Experiment 6.*—This was a repetition of the preceding experiments. The results showed 50 per cent susceptibility in ten animals sensitized with rat blood and later inoculated with a mixture of rat blood and mouse cancer, 77 per cent susceptibility in a series of nine mice sensitized and inoculated with the tumor tissue alone, and 80 per cent susceptibility in the control series of ten normal control mice inoculated with the mixture of rat blood and mouse cancer.

The results obtained in the foregoing experiments are presented in Table I and Text-fig. 1.

TABLE I.

Experiment No.	Group A.*	Group B.	Group C.
1	62.5 per cent immunity (8 mice).		0.0 per cent immunity (8 mice).
2	60.0 per cent immunity (10 mice).		10.0 per cent immunity (10 mice).
3	60.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	10.0 per cent immunity (10 mice).
4	50.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).
5	40.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).	0.0 per cent immunity (10 mice).
6	50.0 per cent immunity (10 mice).	23.0 per cent immunity (9 mice).	20.0 per cent immunity (10 mice).

\* Group A was composed of animals sensitized with 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and mouse cancer. Group B was made up of mice sensitized with 0.2 cc. of rat blood and 10 days later inoculated with mouse cancer alone. Group C was made up of mice not sensitized but inoculated with a mixture of rat blood and mouse cancer.

*Histological Study of the Fate of the Cancer-Rat Blood Mixture  
Inoculated into a Sensitized Animal.*

It has been shown that the reaction which takes place about the immunizing injection of mouse blood into a mouse is similar to that about an injection of rat blood in a mouse. The reaction which takes place around a cancer graft in an immunized mouse has been shown to be similar to that which occurs around injected rat blood in a mouse previously sensitized to rat blood. The following experiment was undertaken to supply material for the histological study of the fate of a cancer graft mixed with rat blood when inoculated into a mouse previously sensitized to rat blood.

A series of mice was injected with 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and mouse tumor. These mice were killed in groups at 24 hour intervals up to the 7th day and histological studies made of the grafts. 24 hours after inoculation there was a massive lymphoid and a mild polymorphonuclear reaction about the graft (Figs. 3 and 4). The reaction was still marked on the

Sensitized to rat blood. Inoc. with mixture of rat blood and mouse cancer.	Sensitized to rat blood. Inoc. with mouse cancer.	Not sensitized Inoc. with mixture of rat blood and mouse cancer.
60.0% immune.	Experiment 2.	10.0% immune.
1 +? - -		-? - -
2 - - -		-? - -
3 - - -		-? - -
4 -? - -		-? - -
5 +? - -		-? - -
6 - - -		-? - -
7 -? - -		-? - -
8 -? - -		-? - -
9 -? +?		-? - -
10 + . .		-? - -
60.0% immune.	Experiment 3 0.0% immune.	10.0% immune.
1 -? - -	+? . . .	+? . . .
2 +? - -	+? . . .	+? . . .
3 -? - -	. . . . .	. . . . .
4 +? - -	. . . . .	. . . . .
5 -? - -	. . . . .	. . . . .
6 -? - -	. . . . .	. . . . .
7 +? +? . .	. . . . .	. . . . .
8 +? . . .	. . . . .	. . . . .
9 +? . . .	. . . . .	. . . . .
10 . . . .	. . . . .	. . . . .
50.0% immune.	Experiment 4 0.0% immune.	0.0% immune.
1 +? - -	. . . . .	. . . . .
2 -? - -	. . . . .	. . . . .
3 +? - -	+? . . .	. . . . .
4 +? - -	. . . . .	. . . . .
5 + -? -	+? . . .	. . . . .
6 . . . .	. . . . .	. . . . .
7 . . . .	. . . . .	. . . . .
8 . . . .	. . . . .	. . . . .
9 . . . .	. . . . .	. . . . .
10 . . . .	. . . . .	. . . . .
Weeks 1 2 3	1 2 3	1 2 3

TEXT-FIG. 1. The rate of growth of mouse cancer inoculated in a mixture with rat blood into mice previously sensitized to rat blood, compared to the rate of growth when the cancer alone is inoculated into sensitized mice and the rate of growth when the cancer and rat blood are inoculated into non-sensitized mice.

2nd day, but by the 3rd day it had begun to diminish and the graft was more or less completely destroyed. After this period there was a rapid subsidence of the reaction. The reaction described above is similar to that seen around the cancer graft in an immunized animal.

*Desensitizing Effect of Generalized Doses of X-Rays.*

In a previously reported experiment it was shown that mice rendered potentially immune by the injection of mouse blood could be reduced to a susceptible state by properly regulated exposure to x-rays, administered between the time of the immunizing dose and that of the cancer inoculation.<sup>5</sup> It has likewise been shown that x-rays administered at certain periods after a foreign protein injection desensitize an animal to such an extent that no anaphylactic shock results after a second injection of the protein.<sup>6</sup> These facts have led us to test the effect of x-rays on the immunity resulting from sensitization of mice with rat blood and the subsequent inoculation of mouse cancer mixed with rat blood.

Normal white mice were injected subcutaneously with 0.2 cc. of defibrinated rat blood. These mice and another group of mice which had not been sensitized were then given daily doses of x-rays for 8 days. The dose was governed by the following factors: spark-gap  $2\frac{1}{4}$  inches, milliamperes 10, time 2 minutes, and distance from target 12 inches. 10 days after the sensitizing injection these animals and several normal controls were inoculated with a Bashford adenocarcinoma mixed with defibrinated rat blood. The results from these experiments are given in Table II and Text-fig. 2.

TABLE II.

Experiment No.	Group A.*	Group B.	Group C.
7	10.0 per cent immunity.		20.0 per cent immunity.
8	27.2 " " "	10.0 per cent immunity.	20.0 " " "

\* Group A comprises thirty-two mice sensitized to rat blood, which were given eight exposures to x-rays and were then inoculated with a mixture of rat blood and mouse tumor. Group B was made up of ten mice not sensitized but given the same amount of x-rays as Group A and then inoculated with cancer alone. Group C was composed of twenty normal animals inoculated with a mixture of rat blood and mouse cancer, which had received no previous injection of blood and no x-rays. The total number of mice used in these experiments was 62.

<sup>5</sup> Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

<sup>6</sup> Hussey, R. G., and Murphy, Jas. B., unpublished observation.

Sensitized. X-rayed: 8 body doses. Tumor and blood inoc. 27.2% immune.			Experiment 8. Normal. X-rayed: 8 body doses. Tumor (alone) inoc. 10.0% immune.			Normal. Tumor and blood inoc. 20.0% immune.		
1	-?	-	-	-	-	+	-	-
2	+?	-	+?	-	Died.	+	-	-
3	+	-	+	-	-	+	-	-
4	+?	-?	+	-	-	+	-	-
5	-	-	+	-	-	+	-	-
6	-?	-	+	-	-	+	-	-
7	+	-	+	-	-	+	-	-
8	+	-	+	-	-	+	-	-
9	+?	-	+	-	-	+	-	-
10	+	-	+	-	-	+	-	-
11	+	-	+	-	-	+	-	-
12	+	-	+	-	-	+	-	-
13	+	-	+	-	-	+	-	-
14	-?	-	+	-	-	+	-	-
15	-?	-	+	-	-	+	-	-
16	+	-	+	-	-	+	-	-
17	+	-	+	-	-	+	-	-
18	+	-	+	-	-	+	-	-
19	+	-	+	-	-	+	-	-
20	+	-	+	-	-	+	-	-
21	+?	-	+	-	-	+	-	-
22	+	-	+	-	-	+	-	-
Weeks. 1	2	3	1	2	3	1	2	3

TEXT-FIG. 2. The effect of generalized x-rays on the immunity to transplanted cancer resulting from a local anaphylactic reaction.

*Effect on the Foreign Protein Reaction of X-Rays Administered Locally.*

The foregoing experiments show that mice develop an enhanced refractory state to the growth of transplanted tumor if they are first sensitized with rat blood and inoculated 10 days later with a mixture of rat blood and mouse tumor. Histological examination made at intervals after the tumor inoculation shows that there is a definite local reaction, made up principally of cells of the lymphoid series, which takes place around the tumor cells. This reaction reaches its maximum about 24 hours after inoculation and then gradually sub-

sides. It seems reasonable in the light of previous observations that the cellular elements constituting the reaction play some active part in the mechanism bringing about the refractory state.

On the assumption that the latter statement is true, it would seem to follow that if it were possible to destroy these cells and at the same time not to injure the tumor cells, the refractory state potentially present might be inhibited to some measure. Since it has been shown that the x-rays in moderate amounts have little if any direct effect on the viability of the cells of transplanted tumor when exposures are made directly to the tumor,<sup>7</sup> and since it is well known that the x-rays when properly regulated have a selective destructive action on the lymphoid elements of the body,<sup>8</sup> it was thought possible through these means to effect the purpose stated above.

Normal mice were inoculated subcutaneously with 0.2 cc. of defibrinated rat blood and 10 days later inoculated in the groin with a bit of Bashford adenocarcinoma mixed with defibrinated rat blood. 15 to 20 hours after the inoculation the mice were covered with sheet lead in which an aperture had been made large enough to allow the rays to affect the area around the graft. This area was then exposed to the x-rays in the following dose: spark-gap 8 inches, milliamperes 5, time 4 minutes and 38 seconds, distance from target 8 inches, filtered through 3 mm. of aluminum. For controls to the above experiment, normal mice were inoculated with bits of the same tumor and were exposed to the same amount of x-rays, and other normal mice were inoculated with tumor and rat blood without previous sensitization or after-treatment with x-rays. The results are shown in Table III and Text-fig. 3.

TABLE III.

Experi- ment No.	Group A.*	Group B.	Group C.
9	30.0 per cent immunity.		20.0 per cent immunity.
10	38.0 " " "	10.0 per cent immunity.	20.0 " " "

\* Group A was made up of thirty-one mice, sensitized to rat blood, which were

<sup>7</sup> Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

<sup>8</sup> Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1904-05, xiv, 21.

inoculated after 10 days with a mixture of rat blood and mouse cancer; 20 hours later they were given a local dose of x-rays. Group B was composed of normal mice inoculated with a mouse cancer and 20 hours later given a local dose of x-rays. Group C consisted of twenty normal mice inoculated with a mixture of mouse tumor and rat blood.

Sensitized. Tumor and blood inoc. X-rayed locally 20 hrs. later: 38.0% immune.			Experiment 10. Normal. Tumor (alone) inoc. X-rayed locally 20 hrs. later: 10.0% immune.			Normal. Tumor and blood inoc. 20.0% immune.		
1	+	-	+	-	-	+	-	-
2	-	-	•	•	•	•	-	-
3	+	-	•	•	•	•	•	•
4	-?	-	+	•	•	•	•	•
5	-?	-	•	•	•	•	•	•
6	+	-	•	•	•	•	•	•
7	-?	-	•	•	•	•	•	•
8	+	-	•	•	•	•	•	•
9	•	•	•	•	•	•	•	•
10	+	•	•	•	•	•	•	•
11	•	•						
12	•	•						
13	•	•						
14	•	•						
15	•	•						
16	•	•						
17	•	•						
18	•	•						
19	•	•						
20	•	•						
21	•	•						
Weeks 1	2	3	1	2	3	1	2	3

TEXT-FIG. 3. The effect of x-rays on the immunity to transplanted cancer resulting from a local anaphylactic reaction when the x-rays are administered locally over the cancer graft 20 hours after inoculation.

*Histological Study.*—Twelve normal white mice were inoculated with 0.2 cc. of rat blood, and 10 days later inoculated with rat blood plus a fragment of a Bashford Adenocarcinoma No. 63. 24 hours after the second inoculation a dose of x-rays was given locally over

the area of the cancer implantation. Two of the mice were killed just before the x-rays were given, and two others immediately afterwards. Eight mice were killed, in groups of two, 24 hours, and 3, 6, and 10 days after x-ray treatment.

24 hours after the second inoculation an extensive cell infiltration was found in the area of the subcutaneous tissue where the mixture of heterologous blood and homologous cancer tissue had been inoculated (Fig. 5). The cells participating in the reaction were chiefly of the lymphoid variety, the polymorphonuclear cells being less numerous.

In the specimens taken immediately after the x-rays were given there was a striking reduction of these cells (Fig. 6) in the area which had been thickly infiltrated immediately before the x-ray treatment. How this reduction of the cells is brought about is a matter of conjecture, but it should be mentioned that no necrotic cells were found in the x-rayed area. 24 hours after the x-ray treatment and later the cell infiltration was gradually restored, although it did not become so extensive as it was before.

The temporary suppression of the lymphoid reaction effected by the local dose of x-rays seems to indicate that the removal of these cells is one of the factors which plays a part in allowing the graft to grow in the sensitized animal.

From the two foregoing groups of experiments the conclusion is drawn that it is possible to overcome, to a certain extent, the immunity to cancer resulting from a local anaphylactic reaction, in two ways. In either case we consider that the effect follows the prevention of the local cellular infiltration from taking place or from becoming effective. In the first of these two experiments animals were desensitized so that the second injection of foreign protein did not call forth the local cellular reaction. In the second experiment the lymphocytes taking part in the local reaction were destroyed by x-rays before they had time materially to affect the cancer graft.

#### DISCUSSION.

The foregoing experiments offer further evidence of the hypothesis that the so called immunity to the transplanted cancers of mice depends on a local cellular reaction in which cells of the lymphoid



type play the principal part. The usual method of producing this immunity is through the injection of a quantity of living homologous tissue, which leads to a non-specific immunity, which in turn is directed against a great variety of cancers and sarcomas, as well as against transplanted normal tissue. It has been suggested that this immunity phenomenon is analogous to the so called anaphylactic reaction, but the exact nature of the relation had never been demonstrated. The experiments reported here indicate that this relation is quite close. The first injection prepares or sensitizes, and the second injection of the cancer cells calls out a cellular exudate such as is observed in local anaphylactic reactions. Why living cells are necessary for the sensitizing dose is not evident, unless it requires living cells to sensitize to living cells. The fact is unmistakable, from the experiments reported here, that the condition of local anaphylaxis renders the tissues affected unsuitable for the growth of a cancer graft, and the histological changes which arise correspond with those seen about a cancer graft in an animal immunized by a previous injection of homologous tissue. That the cellular exudate is the essential inhibiting agent is indicated by the fact that when this exudate is prevented from arising or is arrested, the protective effect is either annulled or materially reduced. It is still uncertain whether the desensitization induced by x-ray exposure results in such a general destruction of the lymphocytes that the number left is insufficient to yield the local reaction, or whether it depends on some other factor. Reasoning from the observed fact that x-rays are capable of actual desensitization even to the extent of preventing anaphylactic shock from a second injection of foreign protein, one may well consider whether the failure of immunity under these conditions does not arise from the inability of the desensitized animals to call out the usual cellular exudate. Inasmuch as the local destruction of the cellular exudate is sufficient to annul or reduce the immunity, it is unlikely that it is of the nature of a serum-carried resistance, for the amount of x-rays needed for this purpose is so small and its area of application so limited that it could hardly produce a general effect. In brief, there seems to be no other explanation for the results recorded than that cells of the lymphoid type are capable of preventing the growth of a transplanted cancer when present locally in sufficient

numbers. Hence, we conclude that these cells are an active agent in bringing about the so called immunity condition to transplanted cancer.

#### SUMMARY.

Mice sensitized by an injection of 0.2 cc. of rat blood and 10 days later inoculated with a mixture of rat blood and a transplantable mouse cancer showed a high degree of immunity to the cancer growth, while mice sensitized in the same manner and inoculated with cancer graft with no rat blood showed no immunity. Likewise, non-sensitized mice inoculated with a mixture of rat blood and cancer cells showed no immunity.

Mice sensitized to rat blood and then given a series of doses of x-rays between the time of this injection and the inoculation of the cancer-rat blood mixture showed a suppression of the factors affording protection or immunity, since the cancers grew as well in these animals as in the controls.

Mice were sensitized with rat blood and 10 days later inoculated with a cancer-rat blood mixture. 20 hours after the inoculation when the cellular exudation was at its height, the cells were destroyed by a local dose of x-rays. The degree of immunity was reduced and the cancers grew almost as well as in the controls.

#### EXPLANATION OF PLATES.

##### PLATE 21.

FIG. 1. Subcutaneous tissue of a mouse inoculated with rat blood, 24 hours after the inoculation.

FIG. 2. Subcutaneous tissue of a mouse inoculated with rat blood, 24 hours after a second inoculation of rat blood.

##### PLATE 22.

FIG. 3. Subcutaneous tissue of a mouse sensitized to rat blood, 24 hours after an inoculation with rat blood mixed with mouse tumor.

FIG. 4. The same as Fig. 3, but in higher magnification.

##### PLATE 23.

FIG. 5. Subcutaneous tissue of a sensitized mouse, 24 hours after an inoculation of a cancer-rat blood mixture, showing an extensive cell infiltration.

FIG. 6. Subcutaneous tissue of a mouse which was sensitized with rat blood, then inoculated with a mouse cancer-rat blood mixture, and 24 hours later given a local dose of x-rays. The tissue was removed immediately after the treatment.

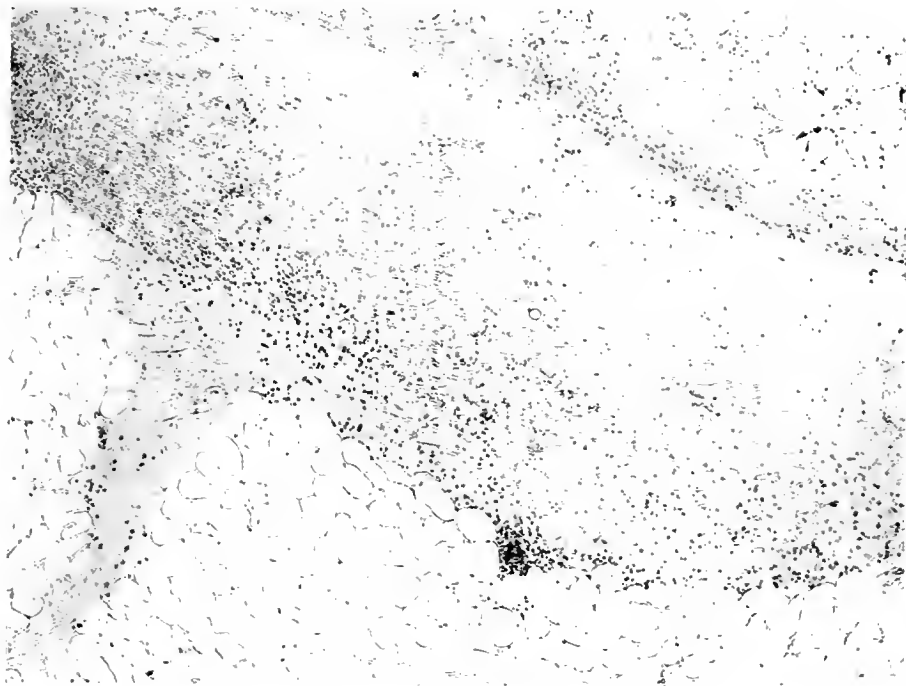


FIG. 1.

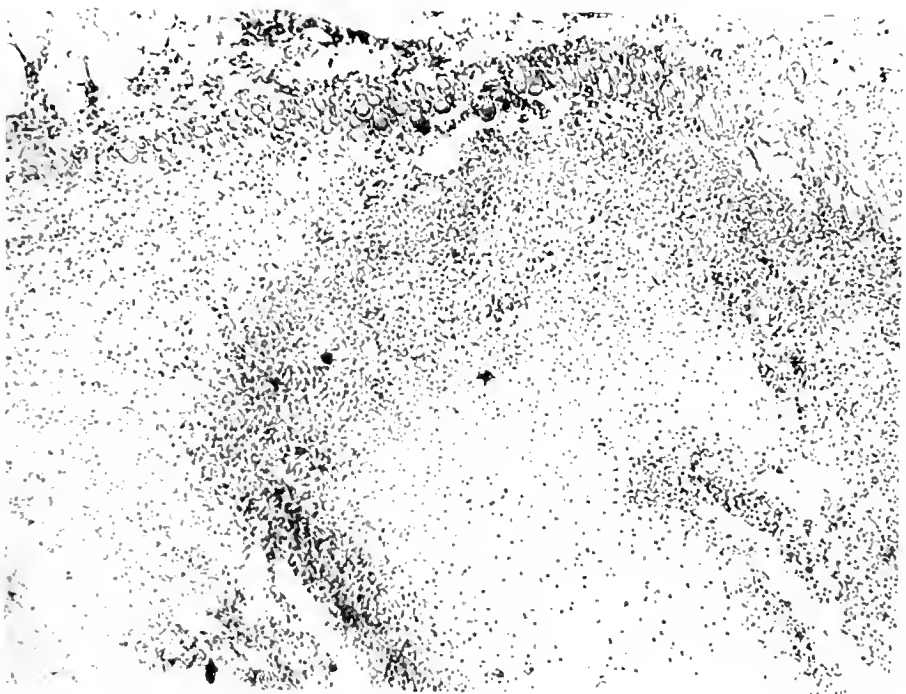


FIG. 2.

(Murphy, Hussey, Sturm, and Nakahara: Cancer grafts. IV.)



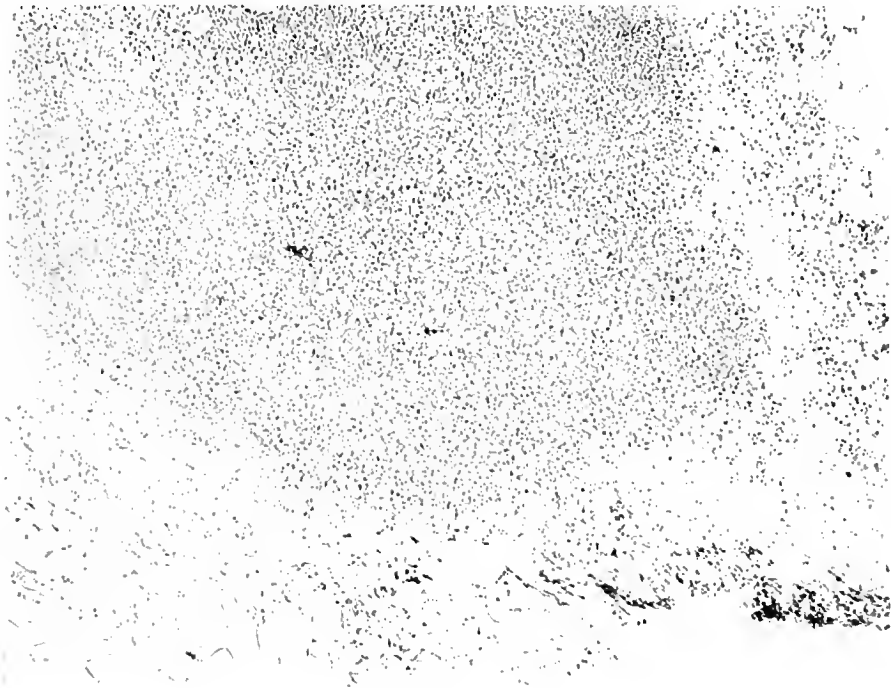


FIG. 3.

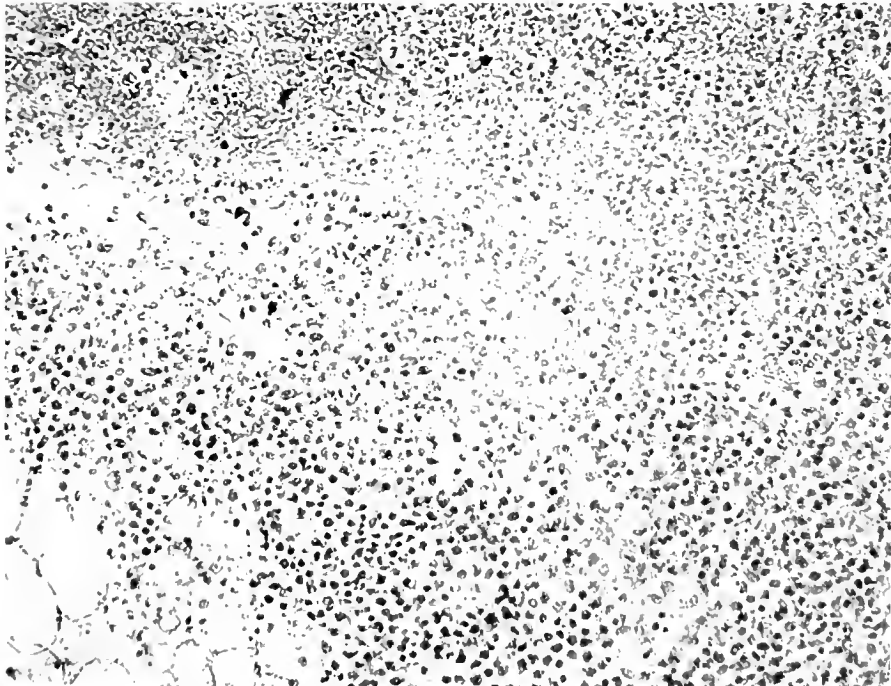


FIG. 4.

(Murphy, Hussey, Sturm, and Nakahara: Cancer grafts. IV)



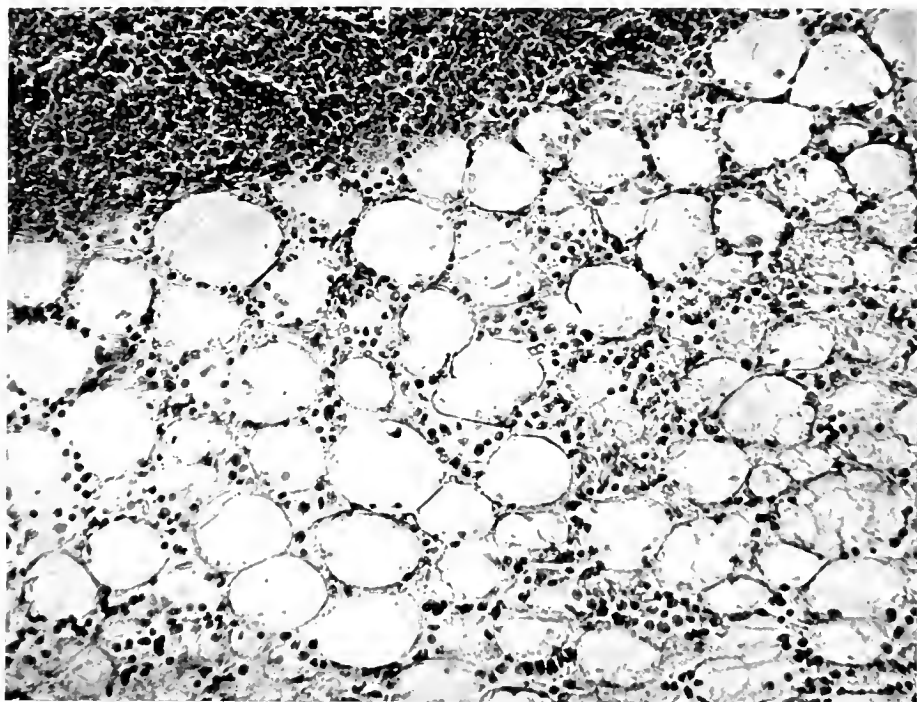


FIG. 5.

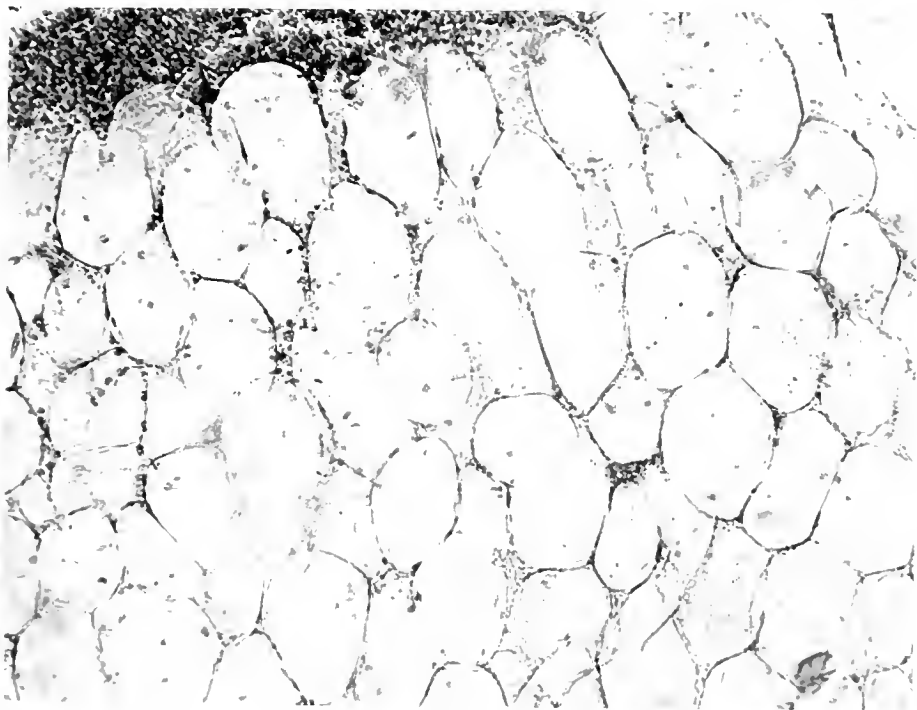


FIG. 6.





# THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

## VI. HISTOLOGICAL COMPARISON OF THE LYMPHOID TISSUE OF NATURALLY IMMUNE AND SUSCEPTIBLE MICE.\*

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PLATES 24 to 26.

(Received for publication, October 22, 1920.)

It has long been known that if a number of normal white mice are inoculated with fragments of transplantable mouse cancer, certain of them will, as a rule, prove to be naturally refractory. The striking histological difference in the reaction about cancer grafts in naturally resistant and in susceptible animals is too well known to be discussed. Murphy and Morton<sup>1</sup> showed that this resistant state is accompanied by a marked lymphocytosis in the blood, absent in the susceptible animals, and also that treatment of normal animals with x-rays destructive to lymphocytes removes at the same time the immunity otherwise present. Hence, the conclusion is drawn that the lymphocyte is a factor in the state of natural immunity.

Histological studies<sup>2-5</sup> paralleling our observations on the circulating lymphocytes<sup>1, 6, 7</sup> indicate that the lymphoid tissue is the basis of the immunity to transplanted cancer induced by intense dry heat,

\* This investigation was carried out by means of funds from the Rutherford Donation.

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

<sup>2</sup> Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

<sup>3</sup> Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

<sup>4</sup> Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

<sup>5</sup> Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

<sup>6</sup> Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

<sup>7</sup> Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

and of that induced by small doses of low penetrating x-rays and by the injection of homologous living cells. Hence, it would appear that a histological comparison of lymphoid organs of naturally immune and naturally susceptible mice to transplanted cancer might give enlightening results, and it was with this end in view that the present study was undertaken.

### *Material and Method.*

Preliminary experiments indicated that more or less definite histological changes take place in lymphoid organs, especially in the spleen, soon after cancer inoculation. These changes are not extensive, but are, nevertheless, of a characteristic nature; hence it is not difficult to determine at the end of the 3rd week of the experiment whether a given spleen comes from an immune or a susceptible mouse. Therefore, we studied the lymphoid organs of young white adult mice inoculated with a given strain of tumor (Bashford Adenocarcinoma No. 63) and killed 3 weeks later. The tissues were fixed in Carnoy's 6-3-1 and stained with eosin-methylene blue, Ehrlich's hematoxylin and eosin, or Heidenhain's iron-hematoxylin. The material consisted of the spleen and lymph nodes from 105 mice, of which 29 came from mice immune to the inoculated cancer, and the remaining 76 from animals with tumors.

### OBSERVATIONS.

The results of histological examination of the lymphoid organs of the naturally immune and susceptible mice are brought out in Tables I and II. It will be noted that the distinction between these two groups is not clear-cut, but that a complete series of intergradations in the changes exists. However, the two groups differ decidedly in the general tendency of the changes, as is brought out in Table III. Descriptions of the nature of the typical changes in lymphoid organs of immune, as contrasted with those of susceptible mice, are given below.

### *Spleen.*

*Immune Mice.*—The general histological appearance of a typically immune spleen is quite characteristic (Fig. 1). The Malpighian bodies are enlarged and show practically no pycnotic cells. Phagocytic cells containing cell fragments found in the germ centers are few, while mitotic figures present in these areas are striking in number (Fig. 2). The superabundance of mitotic figures may be taken to indicate a hyperactivity in tissue proliferation. In typical examples the splenic pulp contains a large quantity of lymphoid tissue which is especially abundant around the vessels. The lymphoid tissue in the pulp contains a considerable number of mitotic figures, which occur only sparingly in normal splenic pulp. Comparatively few degenerating cells with pycnotic or fragmental nuclei are found. The pulp space contains a very small amount of blood, and the deposits of pigment are inconspicuous.

*Susceptible Mice.*—The spleen of a typically susceptible animal presents a different histological picture (Fig. 3). The Malpighian bodies are small and contain very few mitotic figures, while the phagocytic cells with ingested cell fragments are abundant. A few necrotic cells may be found in the peripheral portion of the Malpighian body. The lymphoid tissue of the splenic pulp is very small in amount, and certain vessels may be entirely devoid of this type of tissue. Some of the small perivascular accumulations of lymphoid cells are partly pycnotic. The deposits of pigment are pronounced (Fig. 4), and there is a large quantity of the blood which fills up the spaces unoccupied by lymphoid tissue.

### *Lymph Nodes.*

*Immune Mice.*—Mesenteric, inguinal, and often also cervical and axillary lymph nodes were studied. The changes in these nodes are entirely parallel to those found in the spleen, although they are not so striking. In typical examples of immune mice the lymphoid tissue, in the cortex as well as in the medulla, contains many mitotic figures, while there are scarcely any necrotic cells. The pulp spaces are clouded by a large number of lymphocytes (Fig. 5), while other types of cells, such as those of the endothelial group, are not much in evidence.

*Susceptible Mice.*—Compared to the condition of lymph nodes in immune animals, the lymphoid tissue in various nodes in typically susceptible animals shows a striking inhibition of proliferative activity. This may be judged from the scarcity of mitotic figures in the tissue. Some of the cells in the lymph cords are necrotic. Only a few lymphocytes are found in the pulp spaces, the latter being occupied to a great extent by proliferating endothelial cells (Fig. 6).

In addition to the more typical changes already described, it was noted in several instances that plasma cells appeared in large number in the lymph cords. In fact, in such cases, portions of the cords were almost entirely made up of these cells. This change, however, did not appear to be related to the immunity or susceptibility of the animals, as it occurred irregularly. It may also be mentioned that a few susceptible animals showed a considerable number of polymorphonuclear leucocytes in the lymph cords and even in the cortex, and mast cells in the peripheral sinuses.

The descriptions above refer to the extreme types. As might be expected, almost every gradation is encountered between the extremes, for it is well known that various degrees of immunity exist. In the highly resistant animals the tumor grafts are quickly destroyed, while in others only after a period of growth is the cancer overcome. The highly susceptible animals offer little or no inhibitory effect, and the grafted tumor grows at a rate limited only by its growth energy and the ability of the host to supply stroma, while the less susceptible animals show evidences of a definite retarding effect on the rate of growth of the graft.

In order to show the degrees and variations in the points of difference between the histological appearance of the lymphoid tissue of the susceptible and the resistant animals, we have presented in Tables I and II the results of the study of the individual mice. For the spleen we have included the following points: (a) the relative size of the nodules, (b) the amount of lymphoid tissue in the pulp, (c) the number of mitotic figures present, (d) the amount of necrosis of the cells, and (e) the amount of blood and pigment present. For the lymph nodes we have indicated (a) the number of mitotic figures, (b) the degree of necrosis, and (c) the number of lymphocytes and endothelial cells present in the pulp. The material from the indi-

vidual animals was studied and the records were made without any knowledge of whether the tissues came from a susceptible or immune mouse.

TABLE I.  
*Immune Mice.*

Immune Mouse No.	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
1	±*	+	±	-	±	±	-	-	±	±
2	-	±	-	-	±	+	±	-	±	-
3	±	-	±	-	-	±	-	±	+	±
4	+	+	+	-	-	±	-	±	±	±
5	-	±	-	±	+	-	-	±	+	±
6	+	+	+	±	-	-	±	±	±	+
7	±	+	-	±	±	±	±	-	+	±
8	+	+	+	±	-	-	-	-	±	±
9	+	+	±	-	±	±	+	±	±	±
10	+	±	±	-	±	-	±	-	±	-
11	+	+	+	-	-	±	±	-	±	-
12	+	+	±	±	-	±	±	-	±	-
13	+	+	-	-	±	-	+	-	+	-
14	+	+	±	+	±	-	+	-	+	±
15	+	+	±	±	±	±	+	-	±	-
16	+	±	+	±	±	-	+	±	+	-
17	±	±	±	+	±	±	±	-	±	+
18	±	±	±	±	+	±	-	±	-	±
19	+	±	±	+	±	±	±	-	±	±
20	+	±	±	-	-	±	±	±	+	±
21	+	+	+	±	±	-	±	-	±	±
22	+	±	±	±	±	±	±	±	±	+
23	+	±	+	±	±	±	+	-	+	±
24	±	±	±	±	±	±	±	±	+	±
25	+	±	±	-	-	±	±	±	+	±
26	+	±	+	±	±	-	±	±	+	±
27	+	±	±	±	-	-	±	-	+	-
28	±	±	±	±	±	-	±	±	+	±
29	+	±	-	±	-	-	±	-	±	±

\* In the tables + indicates an increase above the normal; ±, approximately normal; -, a decrease below the normal.

TABLE II.  
*Susceptible Mice.*

Susceptible Mouse No.	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
1	±	±	-	-	±	+	-	-	±	+
2	-	±	±	-	-	±	-	-	±	±
3	-	-	-	±	+	+	-	-	±	+
4	-	±	-	±	±	±	-	-	±	±
5	±	-	-	±	+	+	-	±	-	±
6	-	-	-	-	±	+	-	±	±	-
7	-	±	-	-	±	±	-	-	±	±
8	-	-	±	-	-	-	-	-	+	±
9	-	±	±	±	-	±	-	+	±	±
10	-	-	±	±	-	±	-	+	-	-
11	-	±	-	±	±	±	-	+	±	-
12	±	+	±	-	±	-	±	+	±	±
13	+	+	+	±	±	-	±	-	±	±
14	-	-	-	±	+	-	-	+	±	+
15	±	-	-	±	+	±	-	±	-	±
16	-	±	-	±	-	+	-	±	+	±
17	±	±	±	±	-	-	-	+	±	+
18	±	±	±	±	-	-	-	+	+	±
19	-	-	-	+	+	±	-	+	±	+
20	-	-	-	+	+	-	-	±	-	+
21	-	±	-	+	±	-	±	±	-	+
22	±	±	-	±	±	±	-	-	±	±
23	±	±	±	±	-	±	-	±	-	±
24	±	-	-	±	+	±	±	±	±	±
25	-	±	-	±	±	±	-	±	±	±
26	-	-	-	+	+	-	-	-	±	+
27	-	±	±	+	±	-	±	-	±	±
28	±	+	±	±	±	±	±	+	±	+
29	-	±	-	+	±	±	-	±	-	+
30	-	-	-	+	+	±	±	+	±	+
31	-	-	-	±	±	±	-	±	-	+
32	-	-	-	-	±	±	-	±	±	±
33	±	-	-	±	+	-	±	+	+	±
34	±	-	±	+	+	±	±	-	-	+
35	-	±	-	+	+	±	-	+	±	+
36	-	±	-	+	+	±	±	±	-	+
37	±	-	-	+	+	±	±	±	±	+

TABLE II—*Concluded.*

Susceptible Mouse No.	Spleen.						Lymph nodes.			
	Size of nodules.	Lymphoid tissue in pulp.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Blood.	Pigment.	Mitosis in lymphoid tissue.	Necrosis in lymphoid tissue.	Lymphocytes in pulp spaces.	Endothelial cells in pulp spaces.
38	+	—	+	±	+	±	±	+	—	+
39	—	—	—	+	+	±	±	—	±	±
40	±	—	—	±	—	±	—	+	—	±
41	+	±	±	±	—	±	—	+	+	±
42	+	±	±	+	+	±	±	±	—	+
43	±	±	±	+	±	—	—	+	—	+
44	±	±	±	+	+	±	—	±	—	+
45	—	±	—	±	+	±	±	±	±	+
46	±	±	—	+	±	±	—	+	±	±
47	±	±	±	+	—	±	—	±	+	±
48	±	—	—	+	±	±	—	±	±	+
49	—	—	—	+	+	±	—	±	+	+
50	—	—	—	±	+	+	—	—	±	±
51	±	—	—	+	+	±	±	±	±	+
52	±	±	—	+	±	±	—	±	+	±
53	±	—	±	±	±	±	—	±	—	±
54	±	—	—	±	+	+	—	±	±	+
55	±	—	—	+	+	±	—	—	—	±
56	±	±	—	±	+	±	—	±	±	±
57	—	+	—	+	—	—	—	±	±	±
58	±	—	±	±	+	±	±	±	—	±
59	±	—	—	+	+	±	—	±	—	+
60	—	—	—	+	+	+	±	±	±	±
61	±	—	—	+	±	±	±	±	±	+
62	—	±	—	+	±	±	±	±	—	+
63	±	—	±	—	±	±	±	—	—	+
64	—	±	—	+	±	+	±	±	—	+
65	+	—	+	±	±	±	±	±	±	+
66	±	—	—	+	±	±	—	+	—	±
67	±	—	±	±	±	±	±	+	+	±
68	±	±	±	±	—	±	—	±	±	+
69	—	—	—	±	±	±	±	±	—	+
70	±	—	—	±	—	—	±	—	±	+
71	±	—	—	±	—	±	—	±	±	±
72	±	—	±	±	±	±	—	±	—	±
73	—	—	—	+	+	±	—	+	—	±
74	—	—	—	±	+	±	—	±	—	+
75	±	—	—	±	+	+	—	+	—	±
76	—	±	±	±	—	±	±	—	±	±

TABLE III.

*Percentage of Immune Mice and Susceptible Mice.*

Organ.	Points of difference.		Immune mice.	Susceptible mice.
			<i>per cent</i>	<i>per cent</i>
Spleen.	Size of nodules.	+	69	7
		±	24	47
		—	7	46
	Lymphoid tissue in pulp.	+	41	5
		±	55	39
		—	4	56
	Mitosis in lymphoid tissue.	+	28	4
		±	55	30
		—	17	66
	Necrosis in lymphoid tissue.	+	10	39
		±	55	50
		—	35	11
	Blood.	+	7	42
		±	58	38
		—	35	20
	Pigment.	+	4	13
		±	55	69
		—	41	18
Lymph nodes.	Mitosis in lymphoid tissue.	+	21	0
		±	58	36
		—	21	64
	Necrosis in lymphoid tissue.	+	0	26
		±	42	51
		—	58	23
	Lymphocytes in pulp spaces.	+	45	11
		±	52	50
		—	3	39
	Endothelial cells in pulp spaces.	+	10	47
		±	62	49
		—	28	4



In Table III a summary is given of the percentage of the animals in the susceptible and resistant groups showing the various histological changes indicated above.

#### DISCUSSION.

The point to be emphasized in the present study is the difference in the nature of the changes in the lymphoid organs of immune and of susceptible mice. In immune mice there are more or less marked indications of lymphoid hyperplasia, whereas a lymphoid depletion in varying degree tends to arise in susceptible mice. The former condition resembles greatly the histological picture which has been described in animals in which artificial lymphoid stimulation has been rendered.<sup>2-5</sup> The extreme cases of the latter or susceptible type conform in general nature with the condition induced by a large dose of x-rays,<sup>8</sup> although the cellular destruction is far less extensive. The effect is more like that seen after a long exposure to x-rays of low penetration.<sup>4</sup> These findings are in entire agreement with the results of previous experiments in which the importance of the part played by lymphocytes in artificial resistance to transplanted cancer in mice was pointed out.<sup>5</sup> They harmonize also with the observations of Mottram and Russ<sup>9</sup> who have shown that the spleens of rats resistant to the Jensen rat sarcoma tend to show higher lymphocytic content than the spleens of normal animals.

#### SUMMARY.

The lymphoid organs of mice show definite changes after cancer inoculation. In immune mice there is a tendency towards a lymphoid hyperplasia, while in susceptible mice more or less marked depletion of the lymphoid tissue takes place. These changes are evident at the end of the 3rd week after cancer inoculation.

<sup>8</sup> Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1904-05, xiv, 21.

<sup>9</sup> Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917-18, xc, 1.

## EXPLANATION OF PLATES.

## PLATE 24.

FIG. 1. General histological appearance of the spleen of a mouse naturally immune to transplanted tumor. A low power view.

FIG. 2. Germ center of the spleen of an immune mouse, showing numerous mitotic figures (*M*).

## PLATE 25.

FIG. 3. General histological appearance of the spleen of a mouse susceptible to transplanted tumor. A low power view.

FIG. 4. Deposits of pigment in the spleen of a susceptible mouse.

## PLATE 26.

FIG. 5. Lymph node of a naturally immune mouse, showing abundant lymphocytes in the pulp spaces and a few mitotic figures in the lymph cords (*M*).

FIG. 6. Lymph node of a susceptible mouse, showing the proliferation of endothelial cells in the pulp spaces and pycnotic cells in the lymph cords.

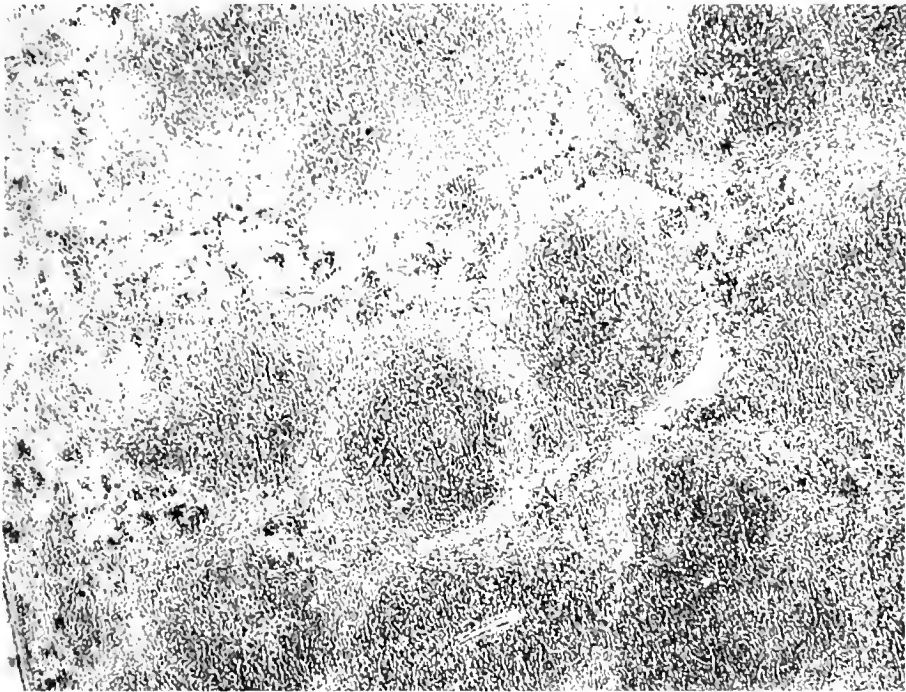


FIG. 1.

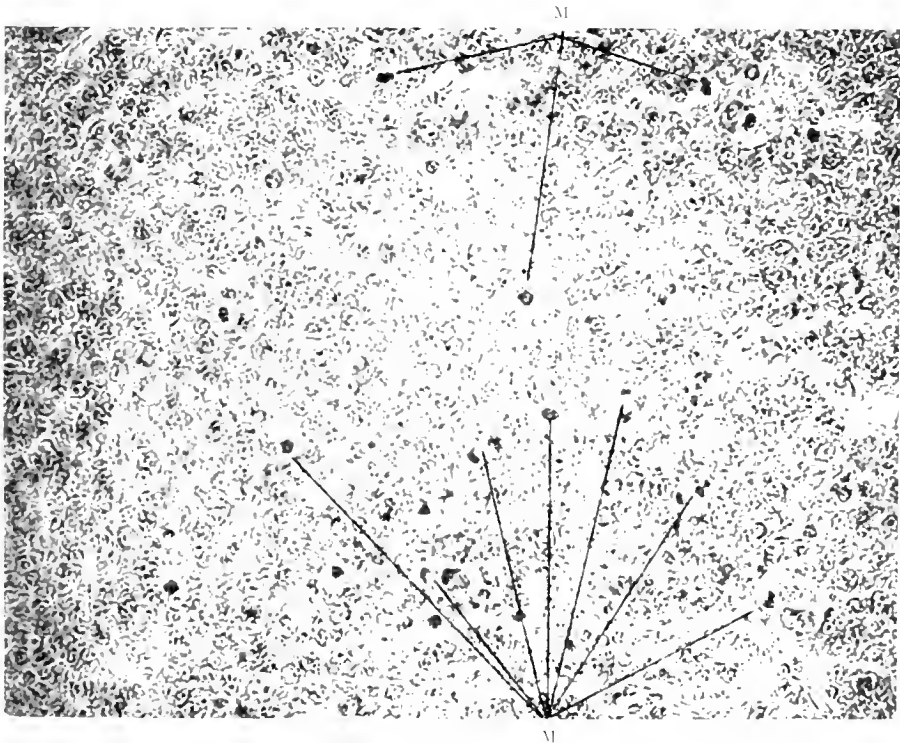


FIG. 2.



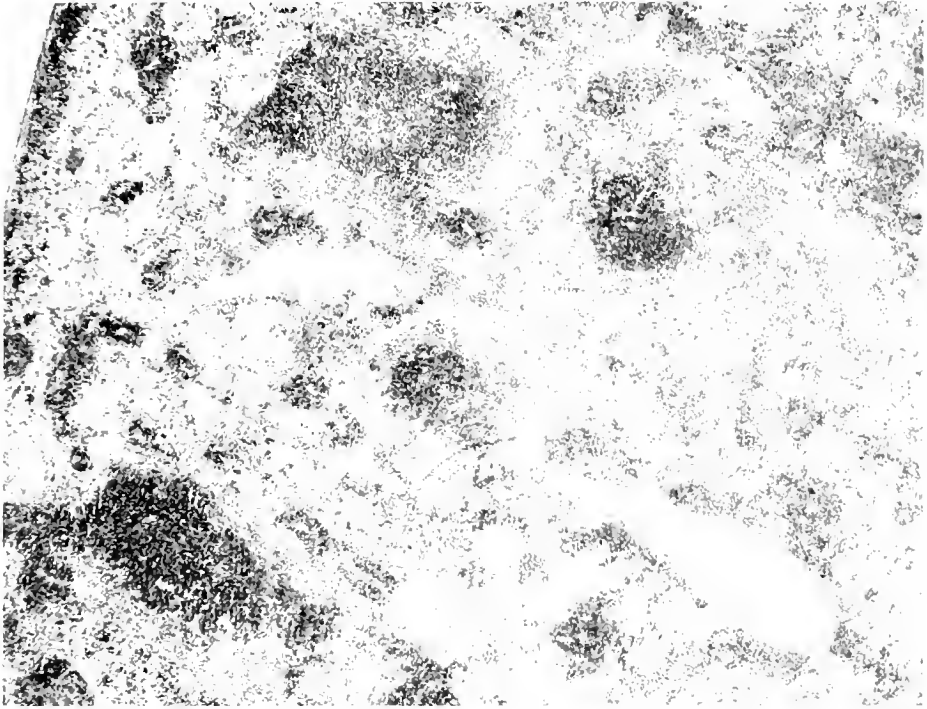


FIG. 3.



FIG. 4.



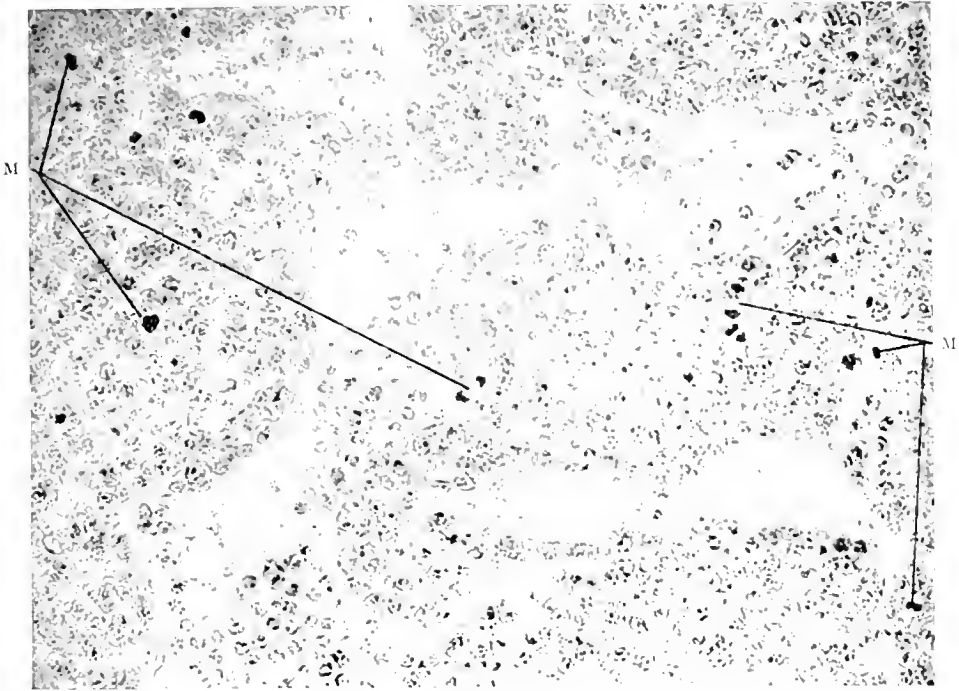


FIG. 5.

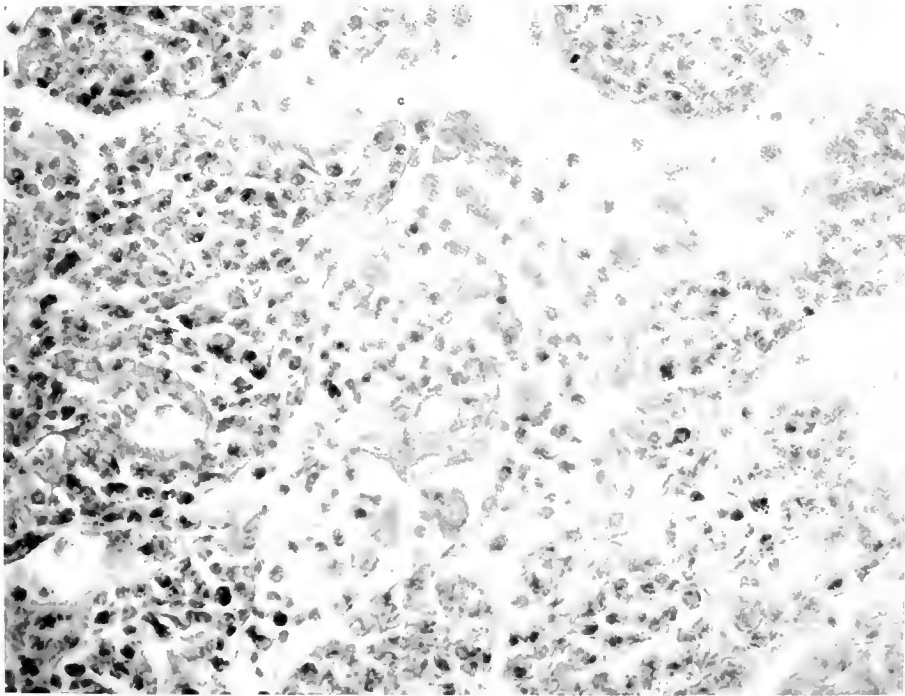


FIG. 6.





# GENERAL LEUCOCYTIC RESPONSE OF THE GUINEA PIG DURING THE REACTION OF ARTIFICIAL IMMUNITY IN EXPERIMENTAL TUBERCULOUS INFECTION.

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(Received for publication, November 8, 1920.)

Since the early studies on immunity reactions it has been known that a definite response of the circulating leucocytes is associated with the process of active immunity. The studies of Zinsser and Tsen,<sup>1</sup> which elaborate Bordet's original observation, furnish experimental evidence of the fact. In clinical medicine, also, it is known that a general leucocytic response accompanies the reaction of the body to bacterial infection. This response is characterized by quantitative changes in the actual number of circulating leucocytes, or in the relative proportion of the various types of cells, or in both.

Despite the fact that these observations are of long standing and are generally accepted, little is known of the actual significance of such changes. It may be stated, however, that for many infections the nature of this phenomenon is taken to indicate the degree of resistance possessed by a given individual. On the other hand, there are some bacterial infections about which there is controversy as to whether there is a characteristic leucocytic response. Hence, there is doubt whether observations of this phase of the blood picture are of value in the experimental or clinical study of such infections. Among these, active tuberculous infections are prominent. The studies of Murphy and his coworkers<sup>2</sup> in this laboratory, and those of Webb, Williams, and Basinger,<sup>3</sup> present evidence to show that the

<sup>1</sup> Zinsser, H., and Tsen, E., *J. Immunol.*, 1917, ii, 247.

<sup>2</sup> Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 35.

<sup>3</sup> Webb, G. B., Williams, W. W., and Basinger, A. F., *Tr. Nat. Tuberc. Assn.*, 1910, vi, 279.

resistance of animals to experimental tuberculous infection is enhanced when artificial lymphocytosis is induced before and after the inoculation of virulent tubercle bacilli. These studies support the observation of several clinicians<sup>4</sup> who have noted in human tuberculous disease that the circulating lymphocytes vary directly with the degree of resistance to the disease manifested by a given individual.

In previous communications from this laboratory, evidence is presented to show that lymphocytosis may be dependent upon the activity of germ centers in lymphoid tissue,<sup>5</sup> and in this sense lymphoid activity may be used as a corollary to lymphocytosis. On account of the interest and importance of these experimental and clinical observations and because the idea suggested by them is not generally accepted, it has seemed worth while to submit the subject to further experiment. We have accordingly made observations on the circulating leucocytes of guinea pigs in which resistance to virulent tubercle bacilli was raised by a previous inoculation of relatively non-virulent tubercle bacilli. We assume that animals so treated are possessed of as great a potential resistance to progressive tuberculous infection as is possible to obtain at present.

#### *Method.*

The method of protecting the animals against virulent tuberculous infection is that first carried out by Trudeau<sup>6</sup> and subsequently elaborated by Baldwin, Krause, and others. A preliminary inoculation of non-virulent tubercle bacilli, Saranac Strain R1, is followed, after a proper interval of time, by an inoculation of virulent tubercle bacilli, Saranac Strain H37.<sup>7</sup> As controls some animals were inoculated with Strain R1 or H37 alone. Guinea pigs were used throughout the observations.

<sup>4</sup> Ullom, J. T., and Craig, F. S., *Am. J. Med. Sc.*, 1905, cxxx, 389. Webb, G. B., and Williams, W. W., *Tr. Nat. Tuberc. Assn.*, 1909, v, 231. Wright, B. L., and King, R. W., *Am. J. Med. Sc.*, 1911, cxli, 852. Solis-Cohen, M., and Strickler, A., *Am. J. Med. Sc.*, 1911, cxlii, 691. Gutstein, M., *Z. Tuberk.*, 1916, xxvi, 336.

<sup>5</sup> Nakahara, W., and Murphy, Jas. B., *Anat. Rec.*, 1921 (in press).

<sup>6</sup> Trudeau, E. L., *Tr. Assn. Am. Phys.*, 1903, xviii, 97.

<sup>7</sup> Acknowledgment is made of our appreciation to the workers at the laboratories of the Trudeau Foundation and Sanatorium for furnishing the cultures of Strains R1 and H37.

Absolute and differential leucocyte counts were made twice weekly on a number of normal guinea pigs over a period of a month. These blood specimens, as well as all others to be referred to, were obtained from a vessel of the ear. For the absolute counts the blood was diluted as usual with 3 per cent acetic acid in a diluting pipette and then shaken. 1 drop of the diluted blood was placed on the disk of a Türk hemocytometer, covered, and allowed to stand long enough for the cells to settle. Eight squares were always counted, and when the deviation of any given square was ten greater or less than the least number of cells obtained for any square, another drop was counted. The diluting pipettes were roughly calibrated before beginning the observations by making comparative counts. The same pipette was used for a given animal for each successive observation. For the differential counts blood films were prepared on cover-slips, then stained with Wright's stain. 300 cells were counted and the percentage of each type determined from this number was applied to the absolute count to estimate the absolute differential count.

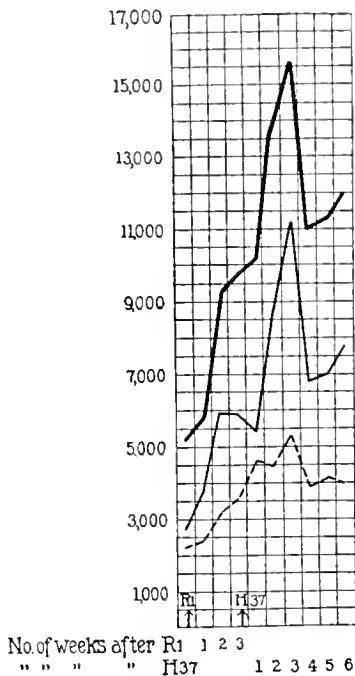
During the period of preliminary observation any animal showing an erratic tendency in the leucocyte count was discarded. The general living conditions were the same for all animals. Therefore, when the experiment was begun, conditions were standardized as far as controllable variables permit, and were maintained throughout the observations.

A number of animals were inoculated subcutaneously into the left groin with 0.2 cc. of a suspension of Strain R1 tubercle bacilli prepared so that a stained film preparation showed not more than ten individual bacilli to a microscopic field. Blood counts were continued in the manner previously described. After about 3 weeks these animals were inoculated with 0.1 cc. of a suspension of the virulent strain, No. H37, and blood counts were continued. This suspension was standardized in the same way as the R1 suspension, except that the number of bacilli was not more than two to a microscopic field.

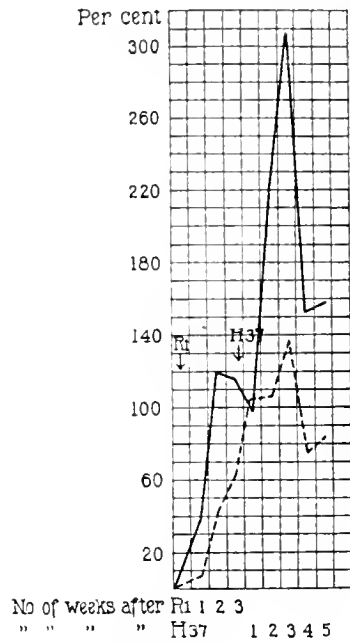
## OBSERVATIONS.

For normal guinea pigs weighing between 200 and 250 gm. the average absolute count was 5,247 leucocytes per cc. of blood. Differential counts showed 52.1 per cent, or 2,734 of these cells, to be lymphocytes and 43.1 per cent, or 2,261, to be amphophils (polymorphonuclear cells).

Following the inoculation of Strain R1 the reaction was characterized by a gradual but definite increase in the total leucocyte



TEXT-FIG. 1.



TEXT-FIG. 2.

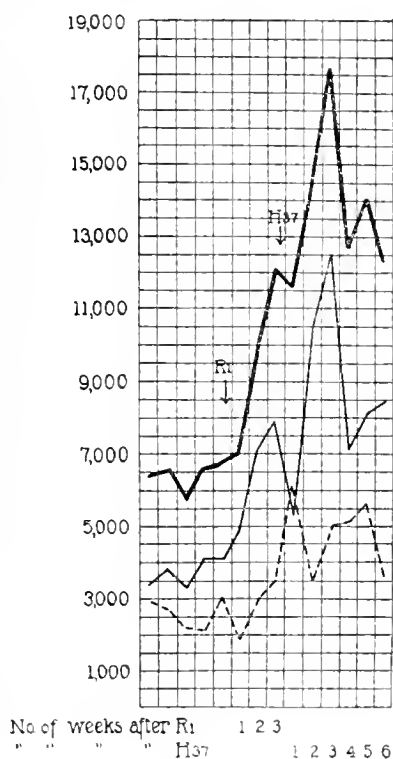
- Absolute count.  
 - - - Lymphocytes.  
 - - - Polymorphonuclears.

TEXT-FIG. 1. Composite absolute and differential white cell counts of all test animals plotted at intervals of 1 week following inoculation and reinoculation.

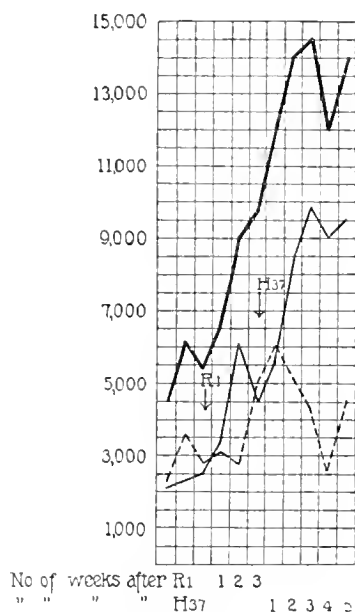
TEXT-FIG. 2. The percentage variation of white cells following inoculation and reinoculation. The standard average\* of 2,734 lymphocytes and 2,261 polymorphonuclears is indicated as zero.

counts (Text-figs. 1 and 2). At the end of 3 weeks, just before the inoculation of Strain H37, this increase was 80.5 per cent, representing an increase in lymphocytes to 60.5 per cent, while the polymorphonuclear cells, though increased absolutely, constituted 34.6 per cent of the white cells, a relative decrease of 8.5 per cent.

The reaction of the protected guinea pigs to the virulent strain, No. H37, was characterized first by a slight fall in the lymphocytes and an



TEXT-FIG. 3.

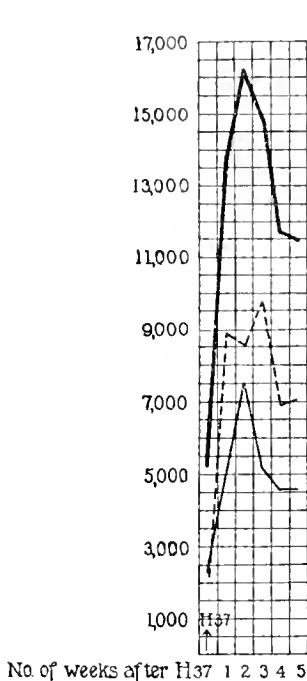


TEXT-FIG. 4.

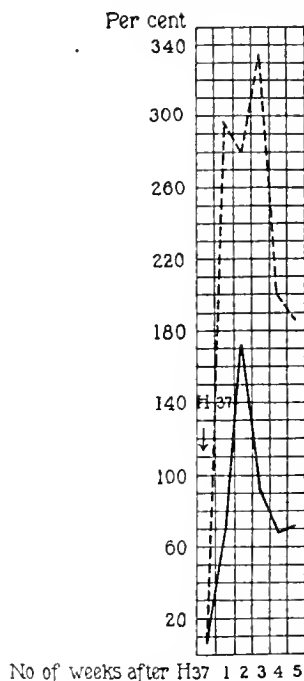
- Absolute count.
- Lymphocytes.
- ..... Polymorphonuclears.

TEXT-FIGS. 3 and 4. Absolute and differential white cell counts of two individuals used in the experiment, plotted at weekly intervals before and after inoculation and reinoculation.

increase in the polymorphonuclear cells. This was observed throughout the 1st week, during which time the animals were obviously quite sick. Then there followed an exaggerated increase in the total leucocyte count, which reached a peak during the 3rd week, at which time the total percentage increase of white cells amounted to 198.5. During this period the lymphocytes increased to a point 306 per cent above their number at the beginning of the experiment, while the polymorphonuclear cells were 9.2 per cent below their normal relative



TEXT-FIG. 5.



TEXT-FIG. 6.

— Absolute count.  
 - - - Lymphocytes.  
 ..... Polymorphonuclears.

TEXT-FIG. 5. Composite absolute and differential white cell counts of individuals plotted at weekly intervals following an inoculation of a heavy suspension of virulent tubercle bacilli.

TEXT-FIG. 6. The percentage variation of white cells in the counts plotted in Text-fig. 5.

average, though numerically they showed some increase. After the peak of the curve was reached, there was a decided drop in the absolute number of white cells, particularly affecting the lymphoid cells. Text-figs. 3 and 4 show typical curves of individual animals used in the experiment. In Text-fig. 4 it will be noted that the normal count shows the polymorphonuclear cells to be greater in number than the lymphocytes, an exception occasionally observed.

Blood counts on the control guinea pigs inoculated with virulent tubercle bacilli alone showed a definite increase in the circulating leucocytes. The relation between the increase of polymorphonuclear and lymphoid elements was erratic. Immediately following inoculation both types of cells increased in about the same numerical proportion. During the 2nd and 3rd weeks the lymphocytes showed a greater increase; after this time there was a progressive decrease in lymphocytes with a corresponding increase in polymorphonuclear cells.

Ten guinea pigs were inoculated with a much heavier suspension of Strain H37. The counts on these animals are shown in Text-figs. 5 and 6. They demonstrate a distinct difference in the reaction to more massive inoculation of virulent tubercle bacilli in contrast to the response to the smaller dose.

#### DISCUSSION.

In the observations recorded it is to be noted that evidence of lymphoid activity varies directly with the resistance to progressive tuberculous infection shown by the animals used. Such facts support the views previously quoted regarding this parallelism. It seems apparent, however, that one is not justified in attempting an explanation of this parallelism since little information is available concerning the function of lymphocytes.

The general idea of some such relation is not new, since as early as 1883 Arloing<sup>8</sup> developed the notion during an attempt to bring experimental evidence to bear on the controversy of that time between clinicians and pathologists concerning the tuberculous nature of scrofula. This investigator and others who followed his lead believed that lymphoid cells possessed some specific property which was

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<sup>8</sup> Arloing, S., *Leçons sur la tuberculose*, Paris, 1892.

active in the defensive mechanism against tubercle bacilli. These opinions were based largely upon observations made on the differences in the reaction of rabbits following the inoculation of material from tuberculous lymphadenitis as compared to that from other tuberculous tissues. Most of this work was done before it was known that there are bovine and human strains of tubercle bacilli, which probably accounted for the observations recorded. Renewed interest came later through the work of Bartel and coworkers who claimed that, while working on experimental tuberculous infection in guinea pigs induced by feeding tubercle bacilli, they observed in lymph nodes in which tubercle bacilli had lodged, without further evidence of invasion, a reaction characterized by a striking lymphoid hyperplasia.<sup>9</sup> Because of the absence of both the characteristic mononuclear phagocyte and the giant cell, they concluded that lymphocytes alone were capable of destroying tubercle bacilli. Further experiments failed to confirm their views. It has been thought that a specific substance might be isolated from lymphoid tissue and used for artificial immunization, but the failure of experiment to support any of these ideas has led to hesitancy in the acceptance of any lead in the study of resistance to tuberculous infection which concerns lymphoid activity.<sup>10</sup> However, we regard the fundamental idea in the studies initiated in this laboratory concerning lymphoid activity in tuberculous infection<sup>2</sup> as totally different from any of those just mentioned. The facts brought out in these observations seem significant, and we believe that they emphasize a probable relation between lymphoid activity and resistance to experimental tuberculous infection which deserves consideration in the study of the factors of resistance to active tuberculous infection in man.

It may be said that several investigators have recorded data which show that the nature of the leucocytic reaction of individuals ill with tuberculous infection in its various forms is analogous to that recorded in this paper. For example, in generalized miliary tuberculosis and in tuberculous meningitis, in which resistance is lowest, since recovery is always improbable, there is a notable deficiency in lymphoid activity as determined by estimations of circulating lymphocytes. In progressive pulmonary tuberculosis this is also the case; on the other hand, in individuals showing an ability to control the disease, there is an increase in the circulating lymphocytes. Although there is no general agreement among various workers that these statements are correct, the disagreement is no more extensive than that concerning the leucocytic reaction in many other infections.

<sup>9</sup> Bartel, J., and Neumann, W., *Centr. Bakt., 1te Abl., Orig.*, 1905-06, **xl**, 518.

<sup>10</sup> Krause, A. K., *Am. Rev. Tuberc.*, 1917-18, **i**, 717.



In this connection it seems of interest to direct attention to certain probable reasons for the differences in opinion; namely, confusion arising from observations made during different phases of a given illness associated with a particular infection, *e.g.* the period of typical manifestations of the infection, that of complications, and that of convalescence; conclusions drawn from data which include too few observations on different individuals; or lack of uniformity in the method of interpretation. These points are illustrated in the statement found in most text-books concerning the leucocytic reaction in general miliary tuberculosis, typhoid fever, and influenza. It is usually asserted that the leucocytic picture in the three infections is similar. However, careful analysis of reports on studies of these infections leads to quite a different conclusion. A summary of the tables in Warthin's paper<sup>11</sup> on general miliary tuberculosis and Thayer's paper<sup>12</sup> on studies on typhoid fever is as follows:

*General Miliary Tuberculosis.*

Absolute count . . . . .	4,128	Decrease . . . . .	2,872 (41.03 per cent)
Polymorphonuclears . . . . .	3,777 (91.5 per cent)	Relative increase . . . . .	20.5 " "
		Absolute decrease . . . . .	1,123 (22.39 " " )
Mononuclears . . . . .	342 (8.3 per cent)	Relative " . . . . .	16.7 " "
		Absolute " . . . . .	1,408 (30.45 " " )

*Typhoid Fever.*

Absolute count . . . . .	5,386	Decrease . . . . .	1,614 (23.05 per cent)
Polymorphonuclears . . . . .	3,323 (61.7 per cent)	Relative decrease . . . . .	8.3 " "
		Absolute " . . . . .	1,577 (32.18 " " )
Mononuclears . . . . .	2,028 (37.6 per cent)	Relative increase . . . . .	12.06 " "
		Absolute " . . . . .	278 (15.89 " " )

The percentage estimations noted are made on the basis of a standard of 7,000 leucocytes per c. mm. of blood, with the percentage relation of polymorphonuclear cells and lymphocytes 70 and 25 respectively.

<sup>11</sup> Warthin, A. S., *Med. News*, 1896, lxviii, 89.

<sup>12</sup> Thayer, W. S., *Johns Hopkins Hosp. Rep.*, 1900, viii, 489.

Further, the recent widespread epidemic of influenza has furnished an opportunity to gain much new information concerning the leucocyte picture found in that infection. Practically all observers affirm a leucopenia as characteristic, but there is difference of opinion regarding the relative proportion of the various cells. Some observers have noted the mononuclear elements to be especially affected,<sup>13</sup> while others pay particular attention to polymorphonuclear elements.<sup>14</sup> The reports which point to the mononuclear elements are based on observations made during the first 5 days of illness, while those which point to the polymorphonuclear cells are concerned with a later period of the illness. In view of the now generally recognized fact that true influenza is of only short duration (1 to 5 days) and that one deals principally with complications in an illness which continues beyond that period, it is suggested that the observations made during the early period should be of greater significance. The report of Olitsky and Gates<sup>15</sup> on experimental influenza lends support to this view. While it is true that all three infections manifest a leucopenia, there are striking differences demonstrated in the degree and character of leucocyte changes.

As further emphasis to the suggestion that more consideration should be given to the possible value of lymphocytes in the defensive mechanism against tubercle bacilli, attention is called to the constant presence of these cells in the local reaction product resulting from tissue injury by tubercle bacilli. Among the numerous investigators who have studied the histogenesis of the tubercle, there has been a tendency by the followers of Baumgarten to disregard the lymphocytes entirely, while those following Metschnikoff have regarded them as progenitors of the characteristic mononuclear phagocytes found in the lesions. It must be stated, however, that all these workers have concerned themselves principally with the controversy as to whether fixed or wandering cells enter chiefly into the reaction.

More recently, however, Wallgren,<sup>16</sup> in a study on experimental tuberculosis of the liver in rabbits, emphasized the part played by

<sup>13</sup> Alder, A., *Folia hæmatol.*, 1919-20, xxv, 16.

<sup>14</sup> Berger, W., *Beitr. Klin. Infektionskrankh.*, 1919-20, viii, 303.

<sup>15</sup> Olitsky, P., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>16</sup> Wallgren, A., *Arb. path. Inst. Helsingfors*, 1911, iii, 139.

lymphocytes in the reaction of the tissues of this organ to tubercle bacilli, while von Fieandt,<sup>17</sup> in a similar study on the meninges, found little evidence of participation by lymphocytes in the process. These observations are of interest in connection with the established fact that different tissues of a host vary in their resistance to parasitic invasion. Soper<sup>18</sup> has demonstrated in the liver of the rabbit a relatively high degree of natural resistance to tubercle bacilli, and it is well known that the meninges are very susceptible to these parasites. Whether or not the success of such a local resistance may be related to a combined activity of the two types of mononuclear cells found in the product of reaction can only be conjectured at this time. Further elaboration of this point must be delayed until methods of experiment are available which will permit its investigation. In the meantime it would seem more plausible to assume that the presence of lymphocytes in these reactions is a purposeful phenomenon, as is believed for other types of cells in various reaction products following tissue injuries. Particularly is this true in the light of available information regarding the factors concerned in the mechanism of immunity in tuberculous infection. There is almost unity of opinion that as far as cellular and humoral elements are concerned, the former are conspicuous while the latter are relatively inconspicuous.

#### SUMMARY.

Guinea pigs have been rendered relatively immune against infection with virulent tubercle bacilli by preliminary inoculation with a suitable quantity of avirulent tubercle bacilli. Blood counts on these animals show that associated with the immune reaction there is a definite general leucocytic response characterized by an absolute increase in the total count, with an absolute and relative increase in the lymphocytes. The period of greatest activity coincides with that known to be the period of greatest reaction, based on anatomical evidence during the course of infection following this method of immunization. Moreover, blood counts made on animals inoculated with avirulent tubercle bacilli alone show an increase in the circulating

<sup>17</sup> von Fieandt, H., *Arb. path. Inst. Helsingfors*, 1911, iii, 235.

<sup>18</sup> Soper, W. B., *Am. Rev. Tuberc.*, 1917-18, i, 385.

lymphocytes during the period of greatest reaction to the infection, while blood counts on guinea pigs inoculated with virulent bacilli alone show an erratic course in which the polymorphonuclear forms are much increased, though not regularly so.

These results indicate a parallelism between lymphoid activity and resistance of the animals to tuberculous infection, and suggest an association of lymphocytes with the factors determining this resistance, a relation which warrants consideration of the blood picture in the clinical study of tuberculous infection.

## GIANT CELLS IN CULTURES FROM HUMAN LYMPH NODES.

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PLATES 27 TO 31.

(Received for publication, December 6, 1920.)

### INTRODUCTION.

In work with cultures from normal and pathological human lymph nodes cultivated either in plasma from the individual from which the node was taken at operation (autogenous plasma, or autoplasma) or in that of another individual (homogenous plasma, or homoplasma), giant cells resembling markedly the giant cells in tuberculous lymph nodes have been found. The explants were from nodes obtained at operations at the Johns Hopkins Hospital, the cultures being made in the usual manner.

Several types of cells migrated out from the explants into the plasma clot or onto the cover-glass; namely, lymphocytes, small wandering cells, large wandering cells, giant cells, spindle and triangular cells, and occasionally large lymphocytes, polymorphonuclear leucocytes, and eosinophils. An account of the migration of the lymphocytes from this series of cultures has recently appeared.<sup>1</sup> The giant cells are described in the present paper. They seemed to be formed, for the most part, within the explant and to migrate out after their formation. They probably either arose from or had the same origin as the large wandering cells which appeared in great numbers in practically every culture. The giant cells and large wandering cells were identical in structure and general appearance, except that the latter were smaller and contained only one or two

<sup>1</sup> Lewis, W. H., and Webster, L. T., Migration of lymphocytes in plasma cultures of human lymph nodes, *J. Exp. Med.*, 1921, xxxiii, 261.

nuclei. The large wandering cells seem to correspond to the epithelioid or endothelioid cells familiar to pathologists. There are at least two possible modes by which the giant cells might have arisen from the wandering cells. The first and probably most generally accepted view is that of the fusion of a few or many large mononuclear wandering cells. Lambert (1912<sup>2,3</sup>) has shown experimentally, by the introduction of lycopodium spores into cultures of spleen, that wandering cells may gather about them and fuse into large foreign body giant cells. He has also shown that such large giant cells may form about other foreign bodies, such as cotton threads, and even the cover-glass appears to act as a foreign body. He found, however, that the connective tissue from the heart of a chick embryo cultivated *in vitro* did not form giant cells about lycopodium spores.

The second possibility of origin for these giant cells is that of division of the nucleus without division of the cytoplasm. There were no signs of mitosis, and although many indications of amitosis of the nucleus were seen in both living and fixed cultures, we were able to observe only one clear case of amitosis of the nucleus without division of the cytoplasm.

The general structure of the giant cells, the arrangement of the nuclei about the central area, and the numerous instances of partial cleavage of nuclei indicate that these cells arise by amitosis from large wandering cells. Since the giant cells form for the most part within the explant it is perhaps not altogether surprising that amitosis is rarely observed.

#### OBSERVATIONS.

There were eight series from normal nodes, one series (No. 19) in autoplasm and seven series (Nos. 19a, 19b, 19c, 10, 13, 15, and 16) in homoplasm. There were 43 cultures of the series in autoplasm, in only one of which was a giant cell observed, and that on the 4th

<sup>2</sup> Lambert, R. A., The production of foreign body giant cells *in vitro*, *J. Exp. Med.*, 1912, xv, 510.

<sup>3</sup> Lambert, R. A., Variations in the character of growth in tissue cultures, *Anat. Rec.*, 1912, vi, 91.

and 6th days. Of the seven series in homoplasma, no giant cells were seen in four (Nos. 19a, 19b, 10, and 15). In Series 19c a few giant cells were found on the 3rd and 4th days. In one of the cultures of Series 13 a single giant cell was observed on the 3rd day and in Series 16 one was first observed on the 6th day.

Three series (Nos. 17, 21, and 22) from chronic lymphadenitis cultivated in homoplasma each showed a few giant cells in several of the cultures, in one series (No. 17) from the 2nd to 7th days, in the second (No. 21) from the 3rd to 11th days, and in the other series (No. 22) a single giant cell was seen on the 3rd day. From one series (No. 3) of chronic lymphadenitis cultivated in autoplasm a no results were obtained.

In one series (No. 1) from acute lymphadenitis in autoplasm several giant cells were seen on the 6th, 7th, and 8th days. Some of them were probably present on the preceding days but the observations were not complete for the earlier period.

The five series (Nos. 9, 7, 18, 14, and 12) from tuberculous nodes all showed giant cells in some of the cultures. In Series 9, explanted in autoplasm, a giant cell was seen as early as the 1st day, and later some of the cultures showed many giant cells. They continued in evidence until the 9th day, when the observation was ended. The other series were cultivated in homoplasma. In Series 7 none was observed on the 1st day but there were many on the 2nd and following days, and a few were still present on the 8th day, the last day of observation. In Series 18 giant cells were observed from the 2nd to 11th days. In Series 12 only a very few were seen from the 3rd to 12th days, and in Series 14 a few were likewise seen from the 3rd to 8th days. Four series (Nos. 23, 23a, 24, and 24a) from caseous tuberculous nodes in autoplasm and homoplasma showed no cell migration.

Two series of cultures (Nos. 5 and 6) from Hodgkin's disease showed a fair number of giant cells. Series 5, in autoplasm, first showed giant cells on the 2nd day; they were fairly numerous up to the 6th day, as long as observations continued. In the other series (No. 6), in homoplasma, two peculiar giant cells were seen 2 hours after the cultures were made. Others were observed from the 2nd to 7th days.

One series (No. 2) from chronic lymphatic leucemia and one (No. 20) from myeloid leucemia gave no results. In one series (No. 8)

from a metastatic sarcomatous node in autoplasm a few giant cells were observed on the 2nd and 4th days. Series 11, in autoplasm, from a metastatic melanotic sarcoma taken at autopsy and Series 4, in homoplasm, from a metastatic round cell sarcoma gave no results.

There were two series (Nos. 25 and 26) from metastatic carcinomatous nodes in homoplasm. No giant cells were seen in either series, both of which were observed from a few hours after explantation up to the 11th day.

To sum up the results of the general observations on the occurrence of giant cells in cultures from normal and pathological nodes, we found that typical giant cells were occasionally seen in cultures from normal nodes and from chronic and acute lymphadenitis. They were very abundant in cultures from tuberculous nodes and fairly numerous in cultures from Hodgkin's disease. A very few were found in cultures from a metastatic sarcomatous node but none from metastatic carcinomatous nodes. The other series were not successful enough to warrant conclusions.

All the giant cells were of the same type, although they varied in size, the number of nuclei, etc. They usually appeared on the 2nd or 3rd day of the cultures after the lymphocytes and polymorphonuclears (if any were present) and wandering cells had migrated out in large numbers into the plasma clot. The wandering cells did not, as a rule, move out into the clot until the lymphocytes and polymorphonuclears were well under way. The wandering cells as they migrated out from the explant into the plasma were usually loaded with bright, greenish, fat globules. Most of the giant cells came out from the explant in this form.

#### *Structure of the Giant Cells.*

The giant cells, with their many nuclei and large accumulation of fat globules, when contrasted with the more familiar cells (Figs. 7 and 10 to 12), were striking objects in the cultures, especially when they were on the cover-glass over a liquefied area. They could be observed much better on the cover-glass than in the plasma clot. Their movements were very slow and deliberate and there was a



certain amount of shifting about of the cytoplasmic contents and of the nuclei; they retained, however, their general internal topography. The giant cells were exactly similar in structure to the large mononuclear wandering cells (epithelioid cells), except that they were larger and contained two or more nuclei. Each giant cell contained a central area about which were arranged the nuclei embedded more or less in a zone of fat globules. Surrounding the zone of fat were many short mitochondrial threads which projected in among the peripheral fat globules and out into the clear ectoplasm. The whole endoplasmic mass was surrounded by a clear, more or less homogeneous ectoplasm.

The various structures are seen to best advantage when the giant cells are flattened out on the cover-glass, as shown in Figs. 1 and 2. They are not always in this form, however, for frequently they hang down from a much smaller area of attachment. In such cells the endoplasmic part with the central area, fat zone, and nuclei may hang far below the cover-glass as a rounded or elongated mass, surrounded by the ectoplasm which spreads out more or less on the cover-glass.

The cells that were flattened out on the cover-slip exhibited a peculiar condition resembling a cell split in half with the cut surface against the cover-glass but covered by ectoplasm. When in this condition the central area seemed to come in contact with the layer of ectoplasm attached to the cover-glass. The zone of fat globules surrounded the rest of the central area like a half shell. The nuclei usually seemed most abundant in a horseshoe area embedded in that part of the fat zone near the cover-glass. In many cells nuclei could not be detected in any other location, but in some instances they were seen scattered more or less throughout the fat zone (Fig. 28), especially in the thicker cells.

*Central Area.*—The central area consisted of a finely granular mass within which could sometimes be seen a clearer homogeneous centrosphere or what appeared to be a centrosphere. No centrioles were observed either in the living or in the fixed cells. In the living cells the fine granules of the central area had a great affinity for neutral red and took up the dye readily and intensely. In fixed specimens this area stained deeply with eosin. The central area varied in size

and under certain conditions occupied the greater part of the endoplasmic area. In the older cultures neutral red granules were sometimes scattered in among the fat globules and were seen in the ectoplasm. Since neutral red is probably only taken up by the non-living cytoplasmic inclusions (Lewis, 1919<sup>4</sup>) this central area must be considered as non-living.

The origin of this central area is not altogether clear. There are several possibilities which must be considered. All cells in tissue cultures gradually accumulate granules which have a great affinity for neutral red. The accumulation usually becomes more marked in the older cultures as the cells become more degenerate. These granules have a decided tendency to become massed about the centriole, usually near one side or at one end of the nucleus. They have been called degeneration granules (Lewis, 1919<sup>4</sup>) and are supposed to represent an accumulation of waste products which the cells are unable to get rid of in their abnormal environment, and come in part perhaps from a breaking down of the cytoplasm. The central area of the large wandering cells and of the giant cells may represent a somewhat similar accumulation of waste products.

Another possibility presents itself. The large wandering cells are voracious phagocytes and are often crammed full of dead lymphocytes in various stages of digestion. The ingested lymphocytes tend to accumulate in what later becomes the central area, and as digestion proceeds they are reduced to granules of various sizes. The giant cells likewise often contain a few partially digested lymphocytes (Fig. 27). As the greater part of this process of ingestion and digestion of lymphocytes seems to take place within the explant, and since the majority of cells that migrate out show only the granular debris which makes up the central area, we have not followed the process in sufficient detail to determine to what extent the central area is dependent upon such digestive waste products for its origin. It seems probable, however, that such waste products are in part responsible for the central area.

These two possibilities are somewhat similar in their nature, both being concerned with the waste products of metabolism. Still another

<sup>4</sup> Lewis, W. H., Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated *in vitro*, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

theory for the origin of these granules suggests itself; namely, that foreign substances, which are not metabolized, enter the cells and are segregated into a gradually enlarging central area.

*Nuclei.*—The nuclei which lay around the periphery of the central area exhibited a peculiar and important arrangement. In the large wandering cells the nucleus was usually at one side of this central area. In the binucleate and trinucleate cells and in giant cells with few nuclei, they tended always to be eccentric as regards the central area (Figs. 3 to 9 and 13 to 25). When there were several or even many nuclei the same arrangement often prevailed, as the nuclei only partially surrounded the central area, and gave a somewhat horseshoe-like effect in most of the cells.

This peculiar arrangement of the nuclei is easily explained through the assumption that the giant cells arise from the large wandering cells by amitotic division of the nuclei without division of the cytoplasm. The first division in a mononuclear cell would result in a binucleate cell with the two nuclei near together but eccentrically placed as regards the central area (Figs. 13 to 16 and 20 to 22). The two nuclei do not always remain near together but may shift more or less around the periphery of the central area (Figs. 5, 16, and 26). As subsequent divisions occur the nuclei gradually surround more and more of the enlarging central area, but even in the multinucleated giant cells there is usually a gap in the circle of nuclei (Figs. 17 to 19 and 27).

It is difficult to understand how such an arrangement of the nuclei about the central area could have come about except by nuclear division, unless the fusion of wandering cells to form the giant cells occurred before the formation of a central area. This is, of course, possible, but since all the large wandering cells that migrate out from the explant possess a similar central area, the fusion, if it does occur, must take place within the explant.

We endeavored to observe nuclear division in these cells after they had migrated out onto the cover-glass, but only one clear case was seen of amitotic division of the nucleus in one of the large wandering cells. The nuclei often became indented more or less, but sometimes straightened out again. In the fixed specimens there were usually many nuclei which showed all stages of amitosis in both the mononuclear forms and the giant cells (Figs. 15 to 22).

The nuclei varied in number from two to 60 or more. Most of the giant cells contained not more than twelve, and only a few had more than twenty-five. The nuclei also varied somewhat in size even in the same cell, probably through unequal cleavage (Fig. 25). Sometimes they were half the usual size or even less. In the latter instances they probably arose as bud-like processes. The cleavage furrow seems either to arise on the side of the nucleus away from the central area, or to be deeper on that side.

We have already noted that the large central area in the large wandering cells and in the giant cells indicates that they have had an abundant food supply of dead lymphocytes, and it is only natural that the great increase in cytoplasmic bulk should be accompanied by a corresponding increase in nuclear material. An important factor, then, in the formation of these giant cells seems to be an overabundant food supply. They are apparently unable to get rid of the waste products of digestion which accumulate in the central area and about which the nuclei arrange themselves.

*Fat Globules.*—The zone of fat globules about the central area is the most conspicuous feature of the living giant cells and of the large wandering cells. The globules are usually numerous and of fairly uniform size. They vary slightly, however, in different cells; in some they seem to be uniformly smaller than in others. In fixed preparations, made with Zenker's fluid without acetic acid, only traces of the spaces which they formerly occupied are to be seen. There is a great difference between the appearance of the living and of the fixed specimens, as may be seen from the figures. The globules stain well after formaldehyde fixation with Scharlach R. In the living cells the globules have a peculiar greenish color.

The zone of fat globules varies considerably in width in different cells and in a few specimens it was reduced to a small area at the poles (Fig. 8). Sometimes the globules seem to have a radial arrangement with reference to the central area. The circumference of the zone is remarkably uniform and even, although the globules are sometimes slightly scattered.

The nuclei are usually embedded in the inner part of the zone where it borders the central area. Mitochondria are scattered among the globules, especially toward the periphery of the zone.

This large accumulation of fat globules depends, we think, upon the abundant supply of food which the cells had while in the explant. They are loaded with fat when they migrate out. The fat may be looked upon as one of the by-products of digestion of the lymphocytes that the giant cells have not been able to use. It may also in part have been ingested as such, and in part have originated as a result of the disordered metabolism in the abnormal environment. It seems to be utilized in some of the cells with still further enlargement of the central area, as shown in Fig. 8.

*Mitochondria.*—The mitochondria were usually abundant in the giant cells in the form of wavy or curly threads. They seemed to be most numerous immediately about the zone of fat globules. There were many between the globules and they extended out more or less into the ectoplasm. Their distribution was similar to that found in the large wandering cells. They were best seen after the cultures had been washed with a solution of Janus green and when the cells were much flattened. It was often difficult to determine how abundant they were in the fat zone. The mitochondria were sometimes remarkably curly; more rarely they appeared as rods or granules. The fact that they were usually long or slender indicates that the cells were in fairly good condition, as in degenerating cells of chick embryo cultures the mitochondria become more or less broken up into rods and granules which later swell to form small vesicles.

*Ectoplasm.*—The ectoplasm consisted of a relatively thick, homogeneous, clear layer and, when the cells were much flattened, formed a broad thin zone (Fig. 29). As already noted, the mitochondria seemed to be most abundant in the part of the ectoplasm immediately surrounding the zone of fat globules. Occasionally, neutral red granules and a few fat globules were seen in the ectoplasm. In fixed specimens it was often noted that the surface adherent to the cover-glass was studded with minute dots or points. This appearance was such as to lead to the belief that the giant cells were attached to the cover-glass by numerous very short processes, or protuberances, and not by a uniform surface. In most of the giant cells that were spread out on the cover-slip the ectoplasm appeared to come directly in contact with the central area on the cover-glass side of the cell, as shown in Fig. 2.

## SUMMARY.

Giant cells resembling those found in tuberculous nodes appeared in cultures of various normal and pathological human lymph nodes cultivated in plasma. They migrated out from the explants from normal and tuberculous nodes, from nodes from acute and chronic lymphadenitis and Hodgkin's disease, and from a metastatic sarcoma. They were most abundant in cultures from tuberculous nodes. The giant cells are similar in structure to the large wandering cells and probably arise from them. We are uncertain, however, as to how the giant cells develop. There is no evidence of fusion of the large mononuclear wandering cells; on the other hand, there is some evidence that they arise by amitosis of the nuclei without division of the cytoplasm, which increases in bulk.

They contain a large central area of more or less granular character which takes up neutral red with great avidity. This central area probably consists of dead material, the waste products of metabolism and of digested foodstuffs such as lymphocytes that the cells are unable to get rid of in the abnormal environment, and perhaps also of accumulated non-living foreign substances that have penetrated into the cells and become segregated.

The central area is surrounded by a conspicuous zone of fat globules in which the nuclei are embedded.

The nuclei vary in number from 2 or 3 to 50 or 60. Usually, however, there are not more than 10 or 20, and in the cells that are flattened out on the cover-slip they often have a horseshoe-like arrangement about the equator of the central area.

Mitochondria are abundant and usually in the form of wavy or curly threads. They seem to be most numerous in the ectoplasm immediately about the fat zone. They also extend in among the fat globules and out into the ectoplasm.

A distinct, clear, homogeneous ectoplasm envelops the cell.

## EXPLANATION OF PLATES.

## PLATE 27.

FIG. 1. Typical giant cell, spread out on the cover-glass, from a living 6 day culture from an acute lymphadenitis specimen in autoplasm (Series 1). The central area, which took neutral red, is surrounded by a zone of fat globules in which are embedded the nuclei. Mitochondrial threads are seen in the clear ectoplasm.

FIG. 2. Idealized section of a giant cell as it lies against the cover-glass.  $\times 750$ .

FIG. 3. Large wandering cell from a living 4 day culture from Hodgkin's node in autoplasm (Series 5). The large central area with the nucleus probably just below it, the fat zone, ectoplasm, and mitochondria partly between fat globules and partly in ectoplasm are shown.  $\times 750$ .

FIG. 4. Another large wandering cell from the same culture on the 5th day. There are indications of a centrosphere in the central area; the nucleus is deeply indented.  $\times 750$ .

FIG. 5. Binucleate large wandering cell from a living 6 day culture from a tuberculous node in homoplasm (Series 7). The small central area, broad fat zone with globules more or less radially arranged, and the mitochondria at the edge of and partly in the fat zone are shown.  $\times 750$ .

FIG. 6. Small giant cell from a living 6 day culture from Hodgkin's node in autoplasm (Series 5). Three nuclei, a large central area, and a fat globule zone surrounded by mitochondria may be seen.  $\times 750$ .

FIG. 7. Giant cell from a living 5 day culture from Hodgkin's node in autoplasm (Series 5). There are four or five nuclei, a small central area, and a broad fat globule zone with mitochondria about its periphery.  $\times 750$ .

FIG. 8. Small giant cell from a living 6 day culture from Hodgkin's node in autoplasm (Series 5). There are four nuclei, a very large central area, only a few fat globules at the poles, and few mitochondrial rods and granules.  $\times 750$ .

FIG. 9. Small giant cell from a living 3 day culture from a tuberculous node in homoplasm (Series 7). There are three nuclei, a small central area, a narrow fat zone, and many long wavy mitochondria in the ectoplasm. The edge of the ectoplasm is rolled up.  $\times 750$ .

FIG. 10. Three lymphocytes from a living 4 day culture from a tuberculous node in autoplasm (Series 9) for comparison with the size of the other cells. Neutral red granules are present.  $\times 750$ .

## PLATE 28.

FIG. 11. Giant cells, wandering cells, and lymphocytes on the cover-glass over the liquefied area, from a fixed and stained 4 day culture from a tuberculous node in autoplasm (Series 9).  $\times 100$ .

FIGS. 12 to 14. Three areas from Fig. 11.

FIG. 12. Very large giant cell with many nuclei surrounding an elongated central area. A few small giant cells and wandering cells may also be seen.  $\times 375$ .

## PLATE 29.

FIG. 13. Giant cells, wandering cells, and lymphocytes.  $\times 375$ .

FIG. 14. Larger giant cells. Elongated migrating lymphocytes are seen on the large giant cell at the right.  $\times 375$ .

## PLATE 30.

FIGS. 15 to 19. Five cells from the same region of the fixed culture shown in Fig. 11, illustrating the arrangement of nuclei as they increase in number, probably by amitosis.  $\times 750$ .

FIGS. 20 to 26. Seven large wandering cells on the cover-glass, from a fixed 2 day culture from a metastatic sarcomatous node in autoplasm (Series 8). Various stages of amitosis are shown. The ectoplasm shows shrinkage.  $\times 750$ .

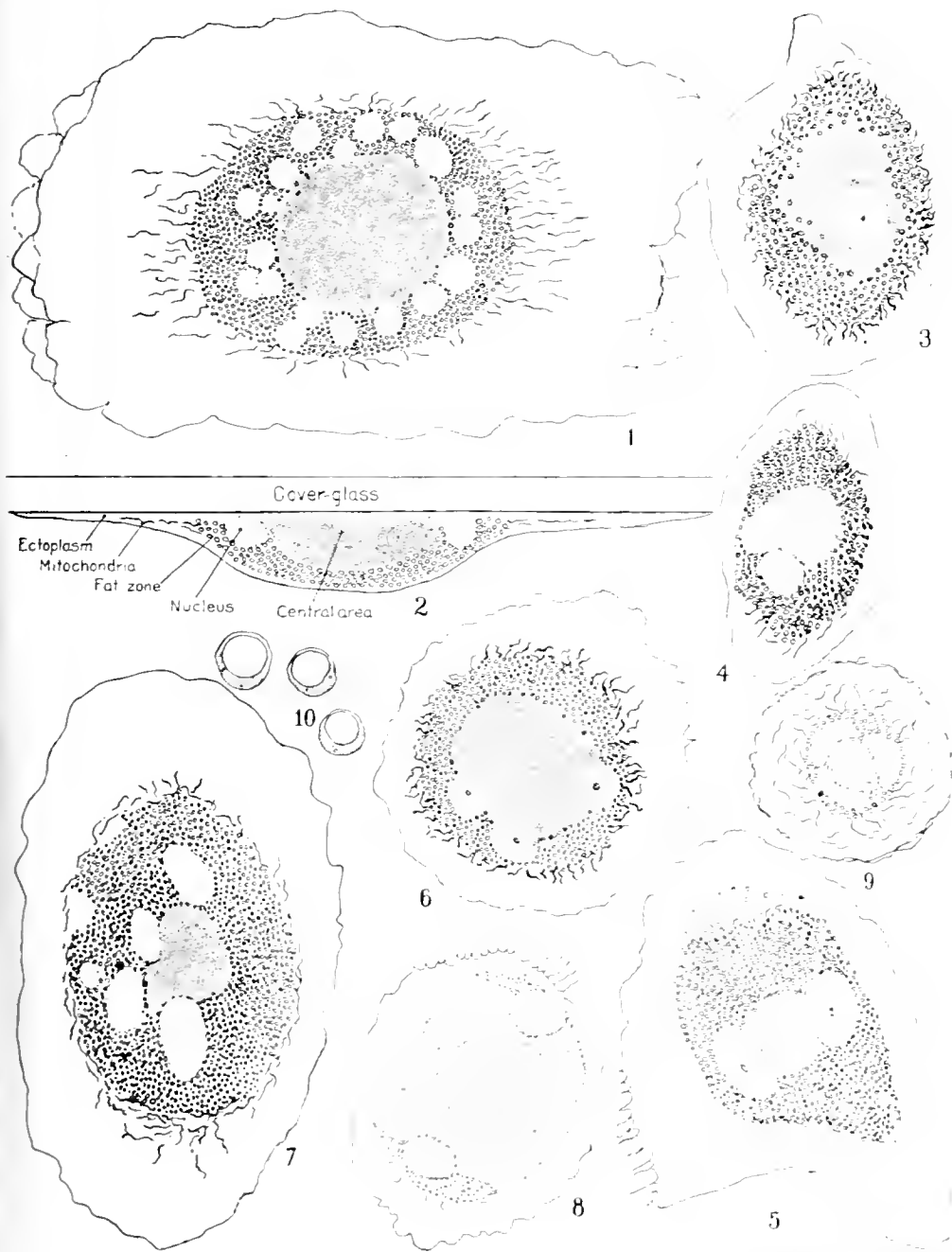
FIG. 27. Giant cell on the cover-glass, from a fixed 8 day culture from a tuberculous node in homoplasm (Series 18). A few incompletely digested lymphocytes may be seen in the central area; the nuclei only partially surround the latter; short mitochondria.  $\times 750$ .

FIG. 28. Giant cell on the cover-glass, from a fixed 5 day culture from a tuberculous node in autoplasm (Series 9). The central area shows a few partially digested lymphocytes; the nuclei are more scattered than usual.  $\times 250$ .

## PLATE 31.

FIG. 29. Giant cell very much flattened on the cover-glass, from a fixed 8 day culture from a tuberculous node in homoplasm (Series 7). Two partially digested lymphocytes may be seen. The ectoplasm is very thin, showing several holes.  $\times 750$ .

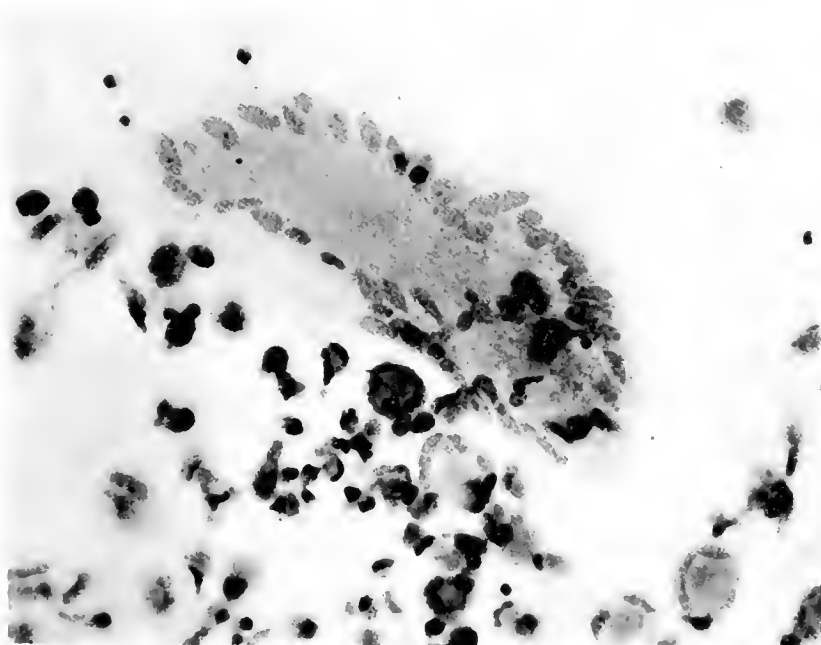








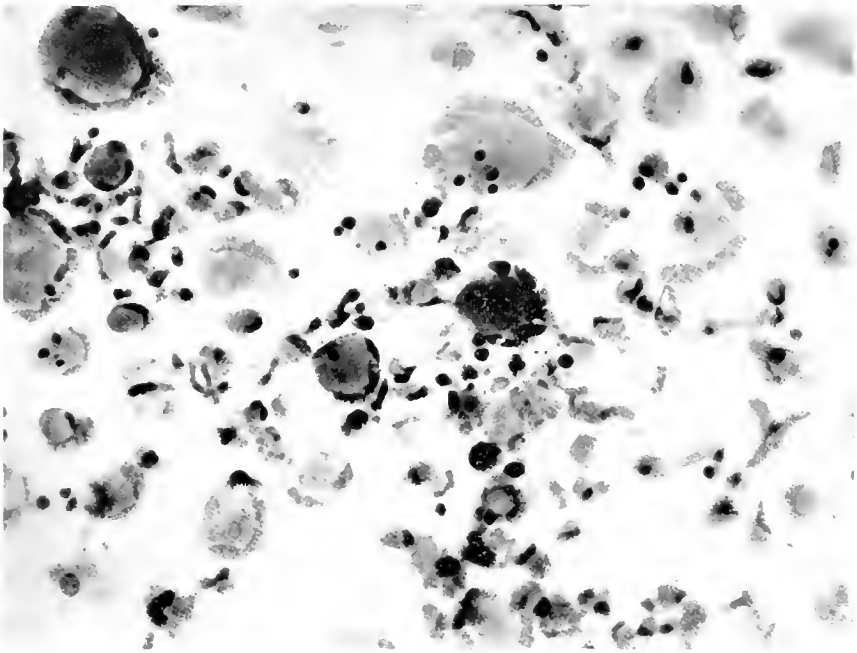
11



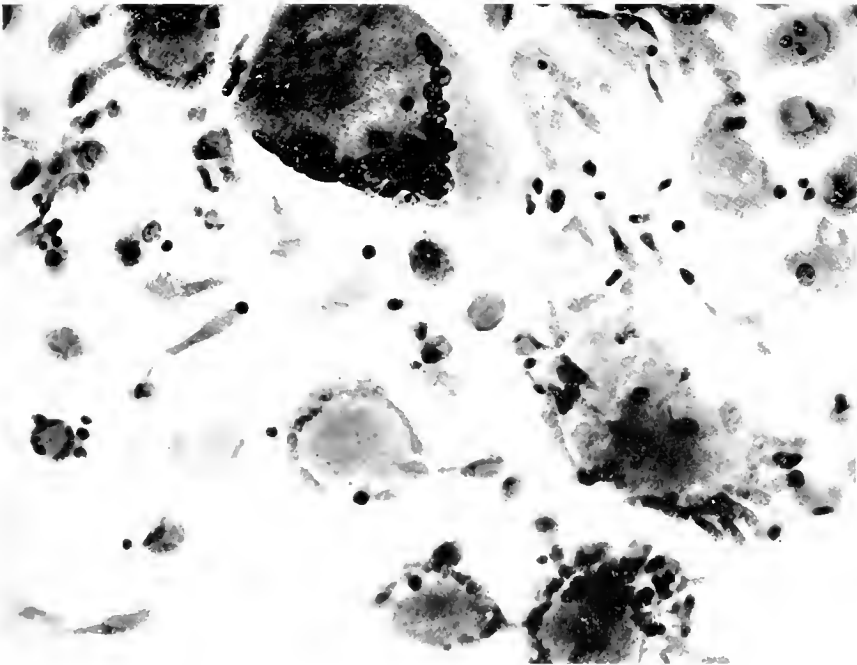
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(Lewis and Webster: Cultures from human lymph nodes.)





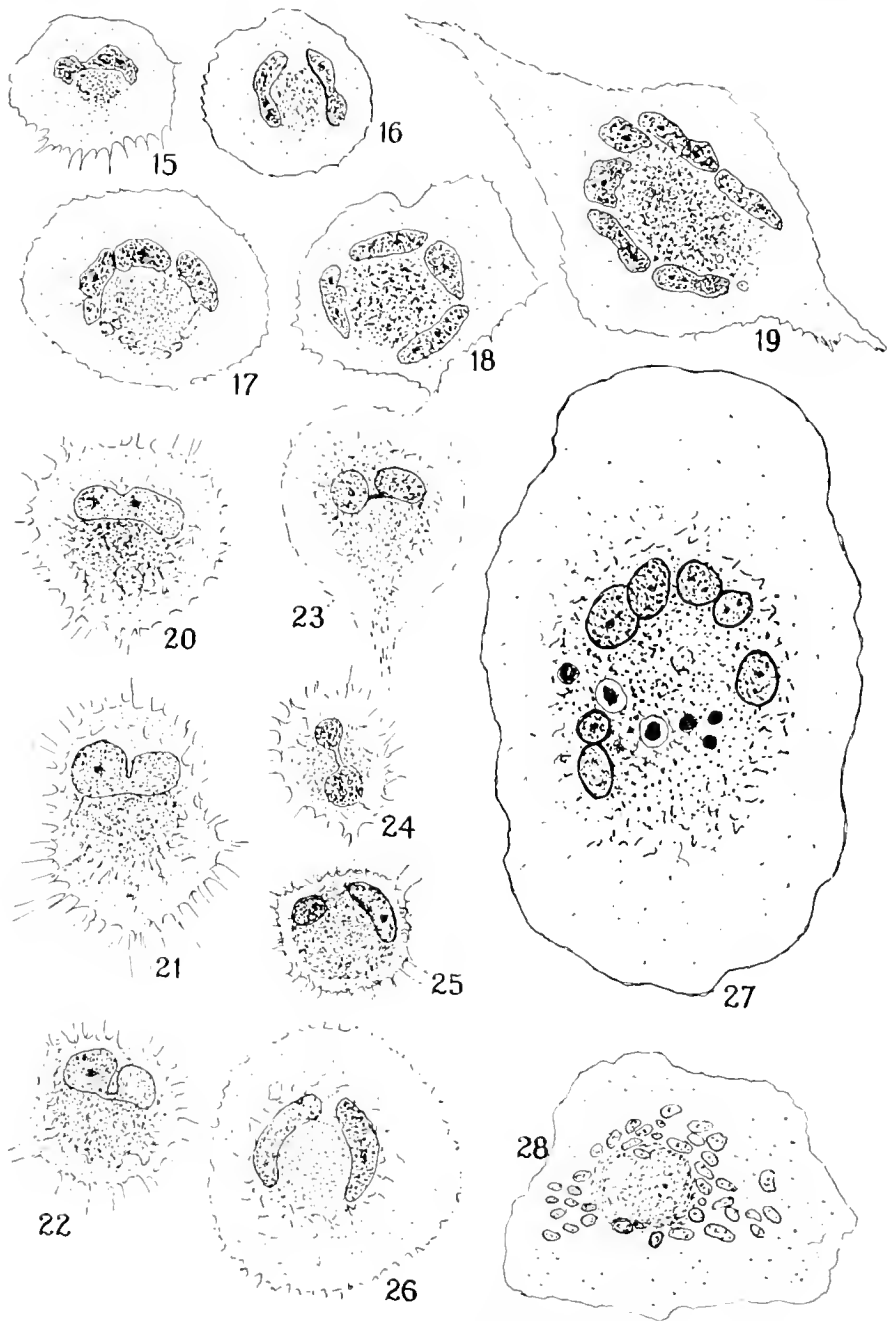
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14

(Lewis and Webster: Cultures from human lymph nodes.)

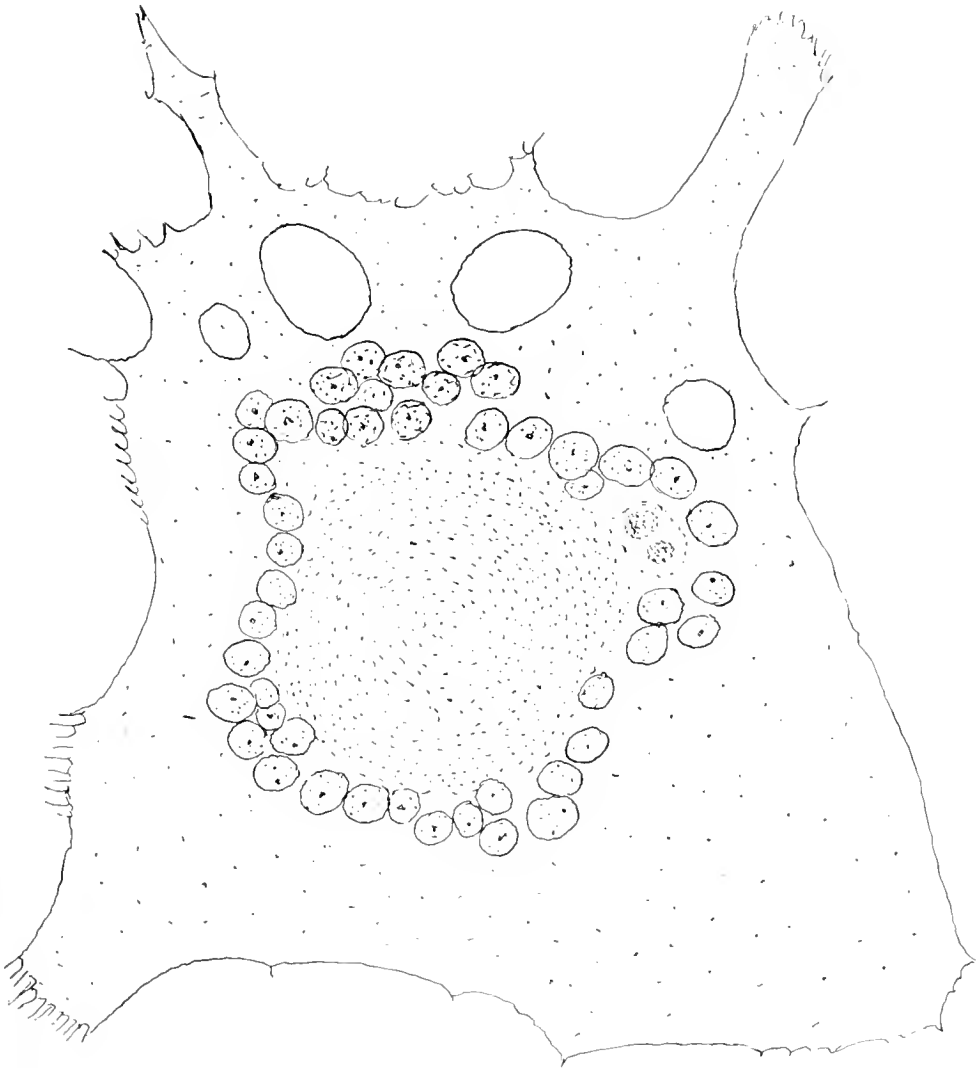




(Lewis and Webster: Cultures from human lymph nodes.)









# EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

## II. FILTERABILITY AND RESISTANCE TO GLYCEROL.

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PLATES 32 TO 34.

(Received for publication, December 13, 1920.)

In our first communication<sup>1</sup> we described the particular effects induced in rabbits by the nasopharyngeal secretions from cases of uncomplicated influenza. We now propose to define more exactly the nature of the peculiar or active substance responsible for these effects and to distinguish it from bacteria of the ordinary species.

Before presenting what are regarded as the decisive experiments, all of which were made in rabbits and guinea pigs, we desire to put briefly on record series of tests carried out on monkeys, *Macacus rhesus*, chiefly, in which the nasopharyngeal secretions from cases of uncomplicated influenza, collected from 12 to 48 hours after the onset of the symptoms, were filtered through Berkefeld V or N candles and injected intratracheally or subconjunctivally, or by both routes, into these animals.<sup>2</sup> In some instances the material was injected as it came from the filter. Occasionally it was concentrated at low temperature *in vacuo* according to the method of Amoss and Taylor.<sup>3</sup>

All these experiments resulted negatively in that no effects were observed which were not also obtained from similarly treated secretions from persons believed not to have suffered from influenza. Hence the work of Nicolle and Lebailly,<sup>4</sup> Gibson, Bowman, and

<sup>1</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>2</sup> All operations were performed under light ether anesthesia.

<sup>3</sup> Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

<sup>4</sup> Nicolle, C., and Lebailly, C., *Compt. rend. Acad.*, 1918, clxvii, 607.

Connor,<sup>5</sup> and Bradford, Bashford, and Wilson<sup>6</sup> was not confirmed by these experiments. But since in many of the monkeys employed any effect produced by the intratracheal injections might have been masked by the presence of lesions of pulmonary tuberculosis, these experiments are not regarded as conclusive. In the meantime the work with rabbits was proceeding in a promising manner and hence the latter animal was chosen for the new series of experiments.

These experiments extended our observations as follows: (1) A condition similar to that found in rabbits injected with the unfiltered nasopharyngeal secretions was obtained by employing filtrates of the lung tissue of such affected animals. (2) Filtrates of the nasopharyngeal washings from early cases of epidemic influenza also induced similar effects in rabbits. (3) When guinea pigs were used instead of rabbits, they showed clinical and pathological effects indistinguishable from those already observed. (4) The peculiar substance inducing these effects, when submitted to the action of 50 per cent glycerol, maintained its activity without alteration in its effects.

#### *Filtered Lung Tissue from Affected Rabbits.*

It was thought advisable to employ at first, for filtration experiments, material possibly more active than the patient's nasopharyngeal secretions. The active substance was therefore carried through several rabbit passages without filtration in the following manner. The nasopharyngeal washings from Case 17, described in the first communication,<sup>1</sup> had been collected 12 hours after the onset of uncomplicated influenza and injected intratracheally in rabbits. As judged by the occurrence of typical effects on the blood and on the lungs, the active material was then transmitted through two successive rabbits by means of the unfiltered lung tissue of each previous animal. The lung tissue of the last rabbit, obtained at the height of the reaction, was ground with sand in sterile saline solution and filtered.

<sup>5</sup> Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1919, i, 331.

<sup>6</sup> Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Brit. Med. J.*, 1919, i, 127.

*Protocol 1.*—The lung tissue of the rabbit corresponding to the third passage of this series was ground with sterile sand in saline solution<sup>1</sup> and the suspension centrifuged at low speed. The supernatant fluid was removed and filtered through a tested Berkefeld candle, size N. 0.5 cc. of the filtrate gave no growth on blood agar plates.

Apr. 17, 1919. 3.5 cc. of the filtrate were introduced intratracheally in a rabbit<sup>7</sup> whose total leucocytes were 17,500, of which 9,625 were mononuclears. Apr. 18. Total leucocytes 10,360, of which 3,936 were mononuclears. The animal had lost 100 gm. in weight; the temperature was unchanged (39.1°C.); conjunctivitis appeared. Apr. 19. Leucocyte count unchanged; temperature 40.1°C. Killed. All the organs except the lungs were normal in appearance. The lungs were voluminous, edematous, and emphysematous, and in the lower lobes diffuse hemorrhages were noted, in the upper lobes small discrete hemorrhages, especially underneath the pleura, which was apparently unaffected. On section, a blood-stained frothy fluid escaped and the hemorrhages were observed to occupy the depth of the tissues. The trachea, especially in the lower third, showed congestion and small hemorrhages and was covered with mucus.

The microscopic examination (Figs. 1 and 2) confirmed the gross appearance. There were generalized extravasations of erythrocytes into the interalveolar structures and intraalveolar spaces, localized small hemorrhages, extensive edema, and emphysema. In addition, a small amount of cellular exudate was found in the parenchyma, consisting mainly of polymorphonuclear acidophilic and of mononuclear cells, among which were larger cells of the respiratory epithelial type. The bronchi showed exfoliated epithelium, some cells of which were necrotic, and contained a mixture of red cells, coagulated serum, leucocytes, and fibrin in small amount. No growth was obtained on aerobic cultivation of the lung tissue.<sup>8</sup>

This experiment, which is typical of many, shows that the lesions of the lungs and of the circulating blood described in the previous paper as arising in the rabbit from the injection of unfiltered nasopharyngeal secretions from early cases of influenza can be produced by the injection of the filtered extract of the lungs of affected rabbits. The next step was the passage from rabbit to rabbit of the active material contained in the lung tissue by means of successively filtered materials.

*Protocol 2.*—A suspension of the lung of the previous rabbit was filtered through a Berkefeld candle, size V. The filtrate was proved sterile by aerobic cultivation tests and was introduced intratracheally into another series of rabbits. While

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<sup>7</sup> Several rabbits were inoculated, but only the rabbit used for further transmission experiments is described in this paper.

<sup>8</sup> The results of anaerobic cultivation will be reported in a later paper.

tests were made with the filtrate an alternate test with unfiltered lung suspension was carried out.

This consecutive series consisted of ten rabbits, which developed in 24 to 48 hours temperature reaction, conjunctivitis, and leucopenia, involving especially the mononuclear cells. At autopsy the animals exhibited the pathological condition of the lungs described as typical of the action of the influenzal material (Fig. 3). There was no essential difference noted in the action of the filtered as compared with the unfiltered suspensions. Aerobic cultures of the lung tissue in blood and plain dextrose broth were systematically made. As a rule no growth was obtained. But in three instances common bacterial species were found. Thus the fifth passage, in which an unfiltered suspension was used, yielded an indefinite pneumococcus; the seventh and eighth passages, in which filtered suspensions were employed, *B. pyocyaneus*;<sup>9</sup> and the tenth passage made with an unfiltered suspension, an unclassified, small, Gram-negative bacillus of hemoglobinophilic nature. The variety of bacteria appearing in these cultures indicates that they were of accidental occurrence.<sup>10</sup>

The series of experiments suggests that the particular clinical reactions and pathological effects induced in rabbits by the nasopharyngeal washings from early cases of epidemic influenza are due to an active substance which passes through Berkefeld filters and survives successive animal passages of the filtrate.

The preceding experiments were made with material obtained in 1919 and passed through rabbits before filtration. The recurrence of the epidemic of influenza in 1920 afforded another opportunity for the study of material from early cases of the disease in the manner indicated, and for the direct injection into the rabbit of the filtered nasopharyngeal secretions of man.

#### *Filtered Nasopharyngeal Secretions.*

Nasopharyngeal washings, obtained from two patients (Nos. 24 and 26, described in the first paper<sup>1</sup>) who had been ill 36 and 30 hours, were shaken in saline solution and filtered through Berkefeld N candles. The filtrates tested by aerobic culture were sterile. They were inoculated intratracheally into rabbits as follows:

<sup>9</sup> Subsequent tests proved that the Berkefeld candle used in these two passages was contaminated with this organism.

<sup>10</sup> The part played by these aerobic microorganisms will be described in another communication.

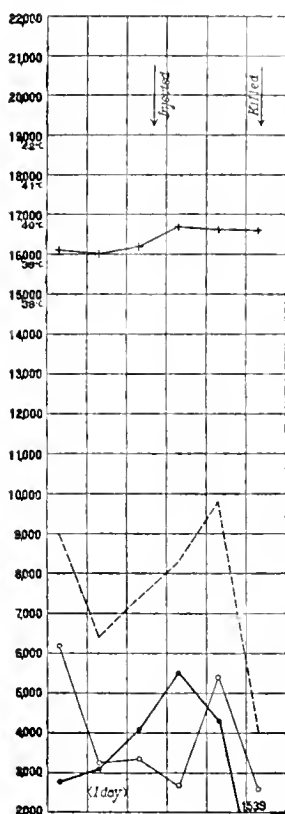
*Protocol 3.*—Jan. 21, 1920. 4 cc. of the filtered nasopharyngeal washings from Patient 24 were introduced intratracheally in a rabbit whose total leucocytes were 10,400, of which 6,760 were mononuclears. Temperature, before injection, 39.2°C. On Jan. 23, after 48 hours, conjunctivitis and a temperature rise to 39.4°C. were noted. The total leucocytes fell to 6,800, of which 4,080 were mononuclears. Jan. 24. Temperature 39.6°C.; total leucocytes 8,200, of which 2,970 were mononuclears. Killed. All the organs except the lungs were normal in appearance. The right lung was more extensively involved than the left. They were both voluminous as a result of edema and emphysema. Small discrete hemorrhages were seen only in the right lung, especially under the pleura. On section the hemorrhages were noted to occupy the depths of the tissues. The bronchi contained mucopurulent material. The microscopic appearance confirmed the gross condition. There were localized small hemorrhages, edema, and emphysema. The capillaries were filled with blood. The small amount of cellular exudate in the parenchyma consisted of polymorphonuclear cells, of respiratory epithelial cells, and, especially in the interalveolar strands, of mononuclear cells. The bronchi showed necrosis and exfoliation of the lining cells and contained coagulated serum, leucocytes, and fibrin. No growth was obtained on aerobic cultivation.

Jan. 24. A second rabbit was injected intratracheally with 3 cc. of the unfiltered lung tissue suspension from the preceding rabbit. Jan. 25. Total leucocytes fell from 11,400 to 9,800, and mononuclears from 5,358 to 4,116. There was conjunctivitis but no temperature rise. Jan. 26. Total leucocytes 9,400, of which 2,256 were mononuclears. Killed. The lesions present in the lungs at autopsy resembled those of the previous rabbit and were regarded as typical. Aerobic cultures showed no growth.

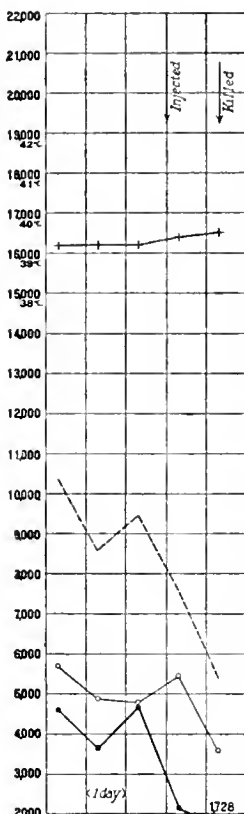
*Protocol 4.*—The typical clinical reaction and pathological effects were induced in rabbits by means of the unfiltered nasopharyngeal secretions from Patient 26. These were described in detail in the first paper.<sup>1</sup>

Feb. 5, 1920. 3.5 cc. of the filtered nasopharyngeal secretions from Patient 26, collected 30 hours after the onset of symptoms, were injected intratracheally in a rabbit whose average total leucocytic count was 7,600, of which 3,331 were mononuclears. On Feb. 8, after 72 hours, total leucocytes fell to 4,050, and mononuclears to 1,539 (Text-fig. 1). Killed. The lungs showed the lesions regarded as typical. No growth was obtained in aerobic cultures.

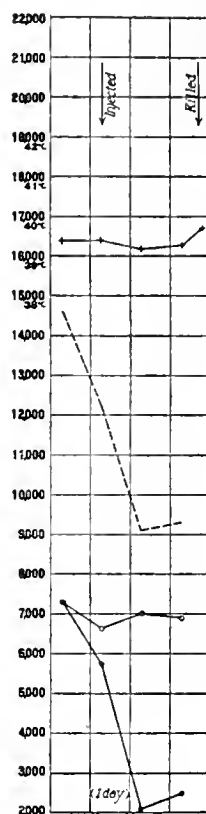
The next passage was effected by employing the lungs of a second filtrate-injected rabbit which reacted typically, but was killed after 48 hours instead of 72. Feb. 7. Rabbit injected with 3 cc. of the unfiltered suspension of these lungs. Feb. 8. Conjunctivitis appeared and temperature rose from 39.2° to 39.4°C. Total leucocytes fell from 9,475 to 7,600, and mononuclears from 4,643 to 2,128. Feb. 9. Temperature 39.5°C.; total leucocytes 5,400, of which 1,728 were mononuclears (Text-fig. 2). Killed. Lungs showed the lesions regarded as typical. No growth obtained in aerobic cultures.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

+—Temperature  
 —x—Total leucocytic count  
 —o—Mononuclears  
 —•—Polymorphonuclears

TEXT-FIG. 1. Effect on the blood count and temperature of the intratracheal inoculation of the nasopharyngeal secretions from Patient 26. First rabbit passage. The rise in the temperature, the leucopenia, and the mononuclear depression 72 hours after inoculation are shown.

TEXT-FIG. 2. Second rabbit passage of material from Patient 26. The rise in temperature, the leucopenia, and the mononuclear depression 24 hours after inoculation are shown.

TEXT-FIG. 3. First rabbit passage of glycerolated material from Patient 6. The temperature is not correspondingly raised as shown in the other text-figures.



The lung tissue of the preceding rabbit of this series of transmissions was stored in 50 per cent sterile glycerol and after 4 months was reinjected into rabbits. The characteristic clinical reaction and pathological effects were then observed in five successive rabbit passages. In all instances no growth was obtained in aerobic cultures. These experiments will be described later.

Protocols 3 and 4 indicate that the filtered nasopharyngeal washings of early cases of epidemic influenza induce in rabbits when injected intratracheally the peculiar changes in the lungs and the blood which have been previously described<sup>1</sup> and are regarded as peculiar and as related to that epidemic disease in man. The first effects of the direct inoculation of the filtrates were noted in 48 hours, but after the first animal passage they were observed at the end of 24 hours. However, it should be noted that while filtrates were employed in the first inoculations the unfiltered lung suspensions were employed in the transfer from rabbit to rabbit. With material from Patient 24 the passages were carried through only two animals, while with material from Patient 26 they were carried through seven successive animals. Moreover, the glycerolated rabbit lung from this series preserved its activity for at least 4 months.

#### *Experiments on Guinea Pigs.*

The next set of experiments was carried out on guinea pigs, the material employed for inoculation being the filtered extracts of the lungs of rabbits which showed the typical lesions.

*Protocol 5.*—Preliminary examination of the first guinea pig gave the following results: total leucocytes 7,900, of which 2,686 were mononuclears; temperature 39.6°C.; weight 750 gm. Apr. 15, 1919, 4.30 p.m. Injected into lungs through trachea 0.65 cc. of the Berkefeld N filtrate (sterile by aerobic cultivation tests) of the suspension of rabbit lung tissue.<sup>11</sup> Apr. 16, 9.30 a.m. Animal sick; temperature subnormal; weight 725 gm.; total leucocytes 13,300, of which 1,862 were mononuclears. Killed. Only the distended lungs showed lesions. The latter consisted of hemorrhages, edema, and emphysema. The whole surfaces and, on section, the interior were mottled with extravasated blood. The microscopic sections showed, besides the escape of blood into the alveoli and bronchi, edema, emphysema, and a certain degree of polymorphonuclear exudation. Aerobic cultures and films showed no bacteria.

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<sup>11</sup> This rabbit represented the second passage of the nasopharyngeal washings from Patient 17, an influenza case described in the previous communication.<sup>1</sup>

*Second Passage.*—The lung tissue of this guinea pig was filtered through a Berkefeld V candle, and 0.5 cc. of the filtrate, sterile by aerobic cultivation tests, was introduced intratracheally into two other guinea pigs. One of the guinea pigs after 48 hours developed a leucopenia and mononuclear depression which persisted for 3 days, and was followed by a gradual return to normal. The other animal, which showed a prompt leucopenia, was killed 24 hours after inoculation. The lung lesions resembled those of the rabbits and the guinea pig of the first passage. No bacteria were obtained on aerobic culture.

The material from the lungs of this guinea pig was suspended in saline solution and injected intratracheally into other guinea pigs as shown in Table I.

TABLE I.

*Results of Intratracheal Inoculation of Guinea Pigs.*

Guinea pig passage.	Material inoculated intratracheally.	Effect on blood.	Remarks.	Lung lesion.
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 4 days.	Allowed to recover.	Typical.
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 2 days.	Killed.	
4th	0.75 cc. of lung tissue from guinea pig of 3rd passage.	Leucopenia, 550 mononuclears, on 3rd day after inoculation.	"	
5th	0.75 cc. of lung tissue from guinea pig of 4th passage.	Leucopenia and mononuclear depression for 2 days.	Allowed to recover.	

The foregoing experiments with guinea pigs indicate that these animals respond to the intratracheal inoculation of materials derived from cases of acute influenza and passed through the lungs of rabbits very much as rabbits do. No tests were made in guinea pigs with nasopharyngeal secretions derived directly from man.

*Effect of Glycerol on the Active Agent.*

Since there is a class of pathogenic microorganisms (vaccinia, virus of poliomyelitis, etc.) which is resistant to glycerol, it was decided to test the action of glycerol on the active material, or agent, in the experiments described.

As a routine practice, the lung tissue derived from rabbits was cut into cubes measuring 0.5 to 1 cm., and was placed in sterile 50 per cent glycerol, which was stored in the refrigerator (4°C.). When the glycerolated tissue was to be used for inoculation it was cultured for sterility, rendered free of glycerol by washing four times in normal saline solution, and suspended as previously indicated.<sup>1</sup>

*Protocol 6.*—Total leucocytes of rabbit 12,225, of which 5,624 were mononuclears. Feb. 18, 1919. Inoculated intratracheally with 3 cc. of suspension of 5 day glycerolated rabbit lung tissue, corresponding to the ninth passage of the nasopharyngeal secretions from Case 6, an influenza patient. Feb. 19. Conjunctivitis. Leucocytes decreased to 9,125 and mononuclears to 2,099. Feb. 20. Leucopenia and mononuclear depression continued, as shown in Text-fig. 3. Killed. Lungs showed lesions regarded as typical and no growth was obtained on aerobic culture.

Feb. 20. 3 cc. of the suspension of the lung tissue from this rabbit were injected intratracheally into another rabbit whose normal total leucocytic count was 13,375, of which 6,955 were mononuclears. Feb. 21. Total leucocytes 9,750, of which 4,582 were mononuclears. A loss of 175 gm. in weight accompanied the leucopenia. This condition endured for 1 day, the animal then returning to normal.

Feb. 26. A rabbit was inoculated with 3 cc. of the suspension of rabbit lung tissue corresponding to the second passage of the nasopharyngeal secretions from another case, Patient 11.<sup>1</sup> In 24 hours leucopenia and mononuclear depression occurred, and persisted for 3 days; the rabbit then returned to normal.

These experiments were preliminary and show that a short immersion for 5 days in glycerol does not affect the activity of the agent. Subsequently lung tissues immersed in the 50 per cent glycerol for longer periods, 8 and 18 days, 4, 7, 9, and 10½ months, were tested. Since there is so much similarity in the results obtained they are summarized in Table II.

From Table II it will be noted that the characteristic reaction could be obtained by the use of material immersed in 50 per cent glycerol for periods varying from 8 days to 9 months. In some instances this material was originally derived from filtrates of lung tissue. After recovery from the glycerolated lung tissue the active agent was transmitted through as many as ten successive animals. The lesions in the lungs of a rabbit inoculated with material exposed to glycerol are shown in Fig. 4. Affected lung tissue exposed to the glycerol for 10½ months failed in two series of experiments to yield the agent in an active state.

TABLE II.

*Results of Experiments with Glycerolated Lung Tissue.*

Series No.	Length of exposure to 50 per cent glycerol.	No. of successive rabbits* which showed effects.	Lung lesion.	Effect on blood.	Nature of material.
1	days 18	10	Typical in all.	Leucopenia and mononuclear depression: 1st passage after 72 hrs.; 2nd passage after 48 hrs.; 3rd passage and subsequently after 24 to 48 hrs.	Filtered.
2	mos. 9	3	" " "	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	Unfiltered.
3	10½	0	None.	None.	"
4	10½	0	"	"	"
5	7	3	Typical in all.	Leucopenia and mononuclear depression after 24 to 48 hrs.	Filtered.
6(Lung tissue of rabbit of 1st passage of Series 5 refiltered.)	7	5	" " "	Leucopenia and mononuclear depression within 24 hrs.	"
7	days 8	3	" " "	Leucopenia and mononuclear depression after 24 hrs.	Unfiltered.
8	mos. 4	5	" " "	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	"
9	4	3	" " "	Leucopenia and mononuclear depression within 24 hrs.	Filtered.

\* The number represents, as a rule, the discontinuance of the experiment and not the cessation of the activity of the agent.

It will also be noted that the action on the circulating blood and lungs was detected usually only after 48 to 72 hours in the first passages, and, as a rule, within 24 hours in the subsequent passages.

#### SUMMARY.

An active transmissible agent present in the nasopharynx in early cases of influenza has been found to produce definite and characteristic clinical reactions and pathological effects in rabbits as already described in an earlier publication.<sup>1</sup>

The experiments here reported indicate that this active agent has the following properties.

1. The agent as it exists in the nasopharyngeal secretions in man, and in the lungs of rabbits injected with the human secretions, passes through Berkefeld V and N candles.

2. The filtered material produces the same effects on the circulating blood and on the lungs of rabbits as the unfiltered material.

3. The peculiar effects described as arising in the inoculated rabbit may also be induced in guinea pigs inoculated with the agent.

4. The agent responsible for the reaction on the blood and the lungs of rabbits withstands the action of glycerol in a sterile 50 per cent solution, for periods up to 9 months. The question must be left open at present whether the agent can withstand longer contact with the chemical. In two experiments after  $10\frac{1}{2}$  months contact the agent induced no observable changes in the blood and lungs of rabbits.

#### EXPLANATION OF PLATES.

##### PLATE 32.

FIG. 1. First rabbit passage of filtered material corresponding to the fourth animal passage of the nasopharyngeal secretions from Patient 17 (Protocol 1). The extensive edema, the emphysema, the cellular exudation into the parenchyma, and localized hemorrhages are noteworthy.  $\times 230$ .

##### PLATE 33.

FIG. 2. Section from the same rabbit as Fig. 1. This section shows particularly the number of localized small hemorrhages and the hemorrhagic extravasation into the parenchyma.  $\times 230$ .

## PLATE 34.

FIG. 3. The sixth passage of the nasopharyngeal secretions from Patient 17. Rabbit inoculated with filtered material (Protocol 2). The hemorrhages and the voluminous condition of the lungs resulting from edema and emphysema are noteworthy. Natural size.

FIG. 4. Rabbit inoculated with the lung tissue shown in Fig. 3 after exposure to 50 per cent glycerol for 18 days. The hemorrhages, voluminous condition of the lungs resulting from the edema and emphysema, and the absence of pneumonic consolidation are shown. Natural size.

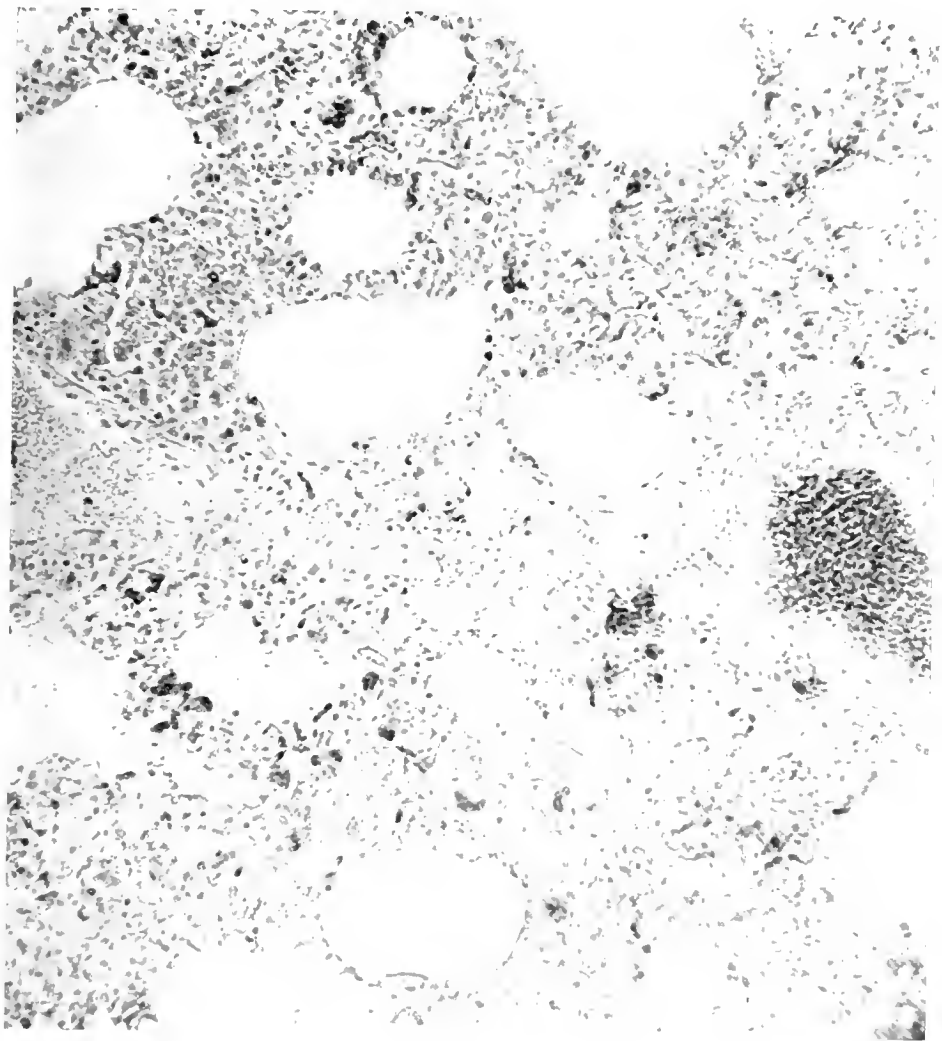


FIG. 1.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. 11





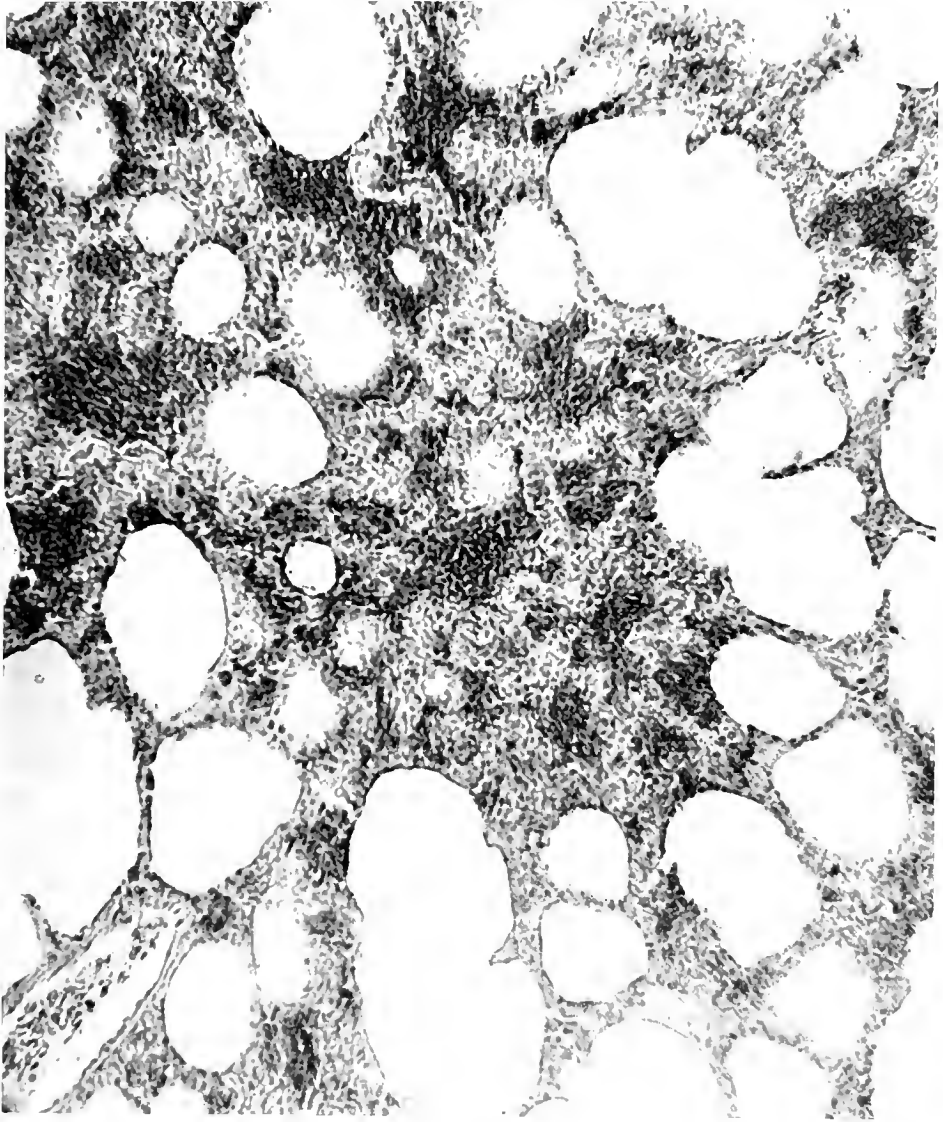


FIG. 2.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. II.)



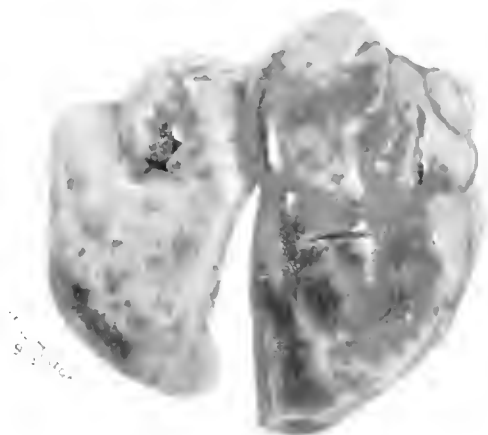


FIG. 3.

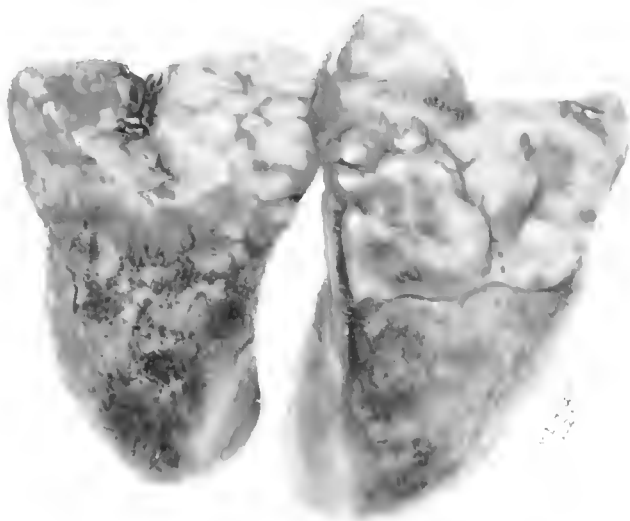


FIG. 4.



## EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

### III. STUDIES OF THE CONCURRENT INFECTIONS.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 35.

(Received for publication, December 13, 1920.)

In two previous communications<sup>1,2</sup> the effects produced in the lungs and blood of rabbits through the intratracheal injection of the nasopharyngeal secretions from patients in the early stages of uncomplicated epidemic influenza were described and traced to a living substance not of the nature of ordinary bacteria. Nevertheless, ordinary bacteria were occasionally, if rarely, encountered in the lungs in the course of the experiments, in which case the lesions of the lungs and the composition of the blood were quite different from those observed in the absence of ordinary bacteria. Since we had in view a possible relation of the active substance giving rise to the peculiar effects observed in the lungs and the blood to the etiological agent of influenza, and since epidemic influenza in man so commonly predisposes to a variety of secondary pulmonary infections, it was also deemed advisable to study the concurrent ordinary bacterial invasions.

The subject was approached from two points of view. First, the circumstances under which ordinary bacteria accidentally invaded the lungs in the course of the transmission experiments were considered, and second, experimental concurrent infections were induced in order to imitate in the rabbit the operation of the predisposing influences of influenza leading to secondary pulmonary affections.

As previously stated, the unfiltered<sup>1</sup> or filtered<sup>2</sup> nasopharyngeal secretions of early cases of epidemic influenza were injected into the

<sup>1</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>2</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

lungs of rabbits by way of the trachea.<sup>3</sup> With unfiltered secretions bacteria present in the nose and throat would, of course, be carried into the lungs; but the bacteria often disappeared or were eliminated in course of subsequent transmission experiments. The kinds of bacteria and the number of times they were observed in the various rabbit and guinea pig passages were as follows:

Pneumococcus Type IV.....	11
"        "    II atypical.....	3
Gram-negative, hemoglobinophilic bacillus .....	2
<i>B. pyocyaneus</i> .....	2
" <i>bronchisepticus</i> .....	2
<i>Micrococcus catarrhalis</i> .....	1
<i>B. coli communior</i> .....	1
<i>Streptococcus viridans</i> .....	1
" <i>hemolyticus</i> .....	1
Gram-negative, slender, spore-bearing bacillus.....	1
Streptothrix.....	1

There was no regularity in incidence or species of the microorganisms found in occasional rabbit passages of a consecutive series. For example, in the filtrate series of ten successive passages from Case 17,<sup>1</sup> the fifth passage yielded *Pneumococcus* Type IV, the seventh and eighth passages *Bacillus pyocyaneus*,<sup>4</sup> and the tenth passage a small, Gram-negative, hemoglobinophilic bacillus. The filtrate series of five such passages of glycerolated material derived from Patient 26 yielded a Gram-negative, long, slender, spore-bearing bacillus in the fifth passage, while the glycerolated series of ten passages of material from Case 17 yielded in the tenth passage *Bacillus coli communior* and a streptothrix. All the remaining passages in these various series were sterile in the ordinary sense. Obviously, therefore, because of this variety the infections may be regarded as of accidental nature. In general these bacteria appeared more often in the earlier passages of unfiltered, and in the later passages of filtered material.

That the appearance of the ordinary bacteria was accidental is shown by single instances of bacterial infection, in several series of

<sup>3</sup> All operations were performed under light ether anesthesia.

<sup>4</sup> The occurrence of *B. pyocyaneus* was the result of faulty technique in filtration.<sup>2</sup>

consecutive animal passages otherwise free. Thus in the glycerolated series of Case 17 only the tenth passage yielded *Bacillus coli communior* and a streptothrix, while the filtrate in the same series showed in the fifth passage only *Pneumococcus* Type IV. Again the glycerolated filtrate series from Patient 26 exhibited in the fifth passage a Gram-negative, spore-bearing bacillus, and the unfiltered nasopharyngeal washings from Case 11 yielded in the third passage *Pneumococcus* Type IV.

In the instances in which aerobic bacteria were encountered in the first passages, the suppression of the bacteria, as already alluded to, could often be effected so that the subsequent passages were free from them. Thus in the transmissions of the nasopharyngeal secretions from Patient 6 the first passage showed *Pneumococcus* Type IV, while in the second, third, and fourth passages no aerobic microorganisms were found. When, therefore, the "influenzal agent," if it may be so designated for purposes of clearness, was present, its peculiar effects could be recognized in the lungs and blood. The suppression of ordinary bacteria was effected as follows: The ordinary bacteria tended to induce consolidation and even abscess of the lung and often remained localized in these areas. The influenzal agent tended to diffuse throughout the lung tissue. By killing the animals early, portions of the lungs could be selected which were free from ordinary bacteria and yet contained the active agent in a transmissible state. Of the seven patients whose nasopharyngeal secretions gave rise to typical effects in rabbits, in four the ordinary bacteria present in the washings were suppressed completely in this manner so that all the animals inoculated subsequently remained free from them.

The clinical condition of the rabbits developing concurrent infections tended to be more grave than that of rabbits in which this form of infection was avoided. The conjunctivitis was purulent instead of catarrhal. There was more loss of weight, and often the animals were prostrated and died in 36 to 48 hours.

The blood showed a greater depression of the leucocytes, involving the polymorphonuclear cells and especially the mononuclear cells in the animals which succumbed acutely; in the others an initial depression was followed in 24 to 48 hours by a leucocytosis, usually a polymorphonucleosis.

The pathological appearances also differed. Whereas in the absence of ordinary bacteria the lungs showed hemorrhagic edema and emphysema, without consolidation and pleuritis, in the presence of these bacteria a pneumonic consolidation involving one or more lobes and sometimes actual abscess with softening of the tissues arose. The several kinds of bacteria mentioned were found more or less abundantly in the consolidated areas.

It is obvious, therefore, that the effects of materials derived from cases of influenza in man on the lungs and the blood of rabbits are sharply distinguished according as common kinds of bacteria do or do not multiply in the lungs. Under the former circumstances the ordinary and usually severe inflammatory reaction follows; under the latter conditions a peculiar hemorrhagic edema of patchy character arises in the lungs, and the blood shows a marked and characteristic leucopenia with mononuclear depression.

#### *Experimental Concurrent Infection.*

It has been commonly and widely noted that cases of influenza in man, uncomplicated by concurrent infections of the bronchi and lungs, tend quickly to recover, but that when these structures are involved in ordinary bacterial infections several kinds of bacteria appear, among which are streptococci, Pfeiffer bacilli, pneumococci, staphylococci, and, rarely, meningococci and even other bacteria; in this event the disease process is correspondingly rendered more intense. The impression is widespread that the inciting agent of influenza, whatever it may be, renders the lung structures more vulnerable to these bacteria, many of which are ordinarily present in the nasopharynx in health. Hence the question at once arose whether the influenzal agent under consideration also predisposed the pulmonary structures to such concurrent or secondary infections. A series of experiments was devised to test this point. The first step was to determine the effects of pure cultures of the several bacteria alone.



*Intratracheal Inoculation of Bacteria.*

The microorganisms of the ordinary kind to which attention was directed were a Type IV and an atypical Type II pneumococcus, *Bacillus Pfeifferi*, and *Bacillus bronchisepticus*, the last because it is a common inhabitant of the upper air passages of the rabbit.

*Pneumococcus*.—The strains of pneumococci employed were freshly isolated from the lungs of rabbits used in the transmission experiments, or directly from the nasopharyngeal washings and sputum from the secondary pneumonias of cases of influenza, or from the normal sputum of man.

If small numbers of the pneumococci were inoculated intratracheally in rabbits, the blood picture remained unchanged for 2 to 4 days, after which a more or less pronounced polymorphonuclear leucocytosis arose which lasted for 2 to 4 days, when the blood returned to normal. Larger doses of the pneumococci led to a prompt polymorphonucleosis, a bacterial invasion of the blood stream, and death. The lesions present in the lungs and pleura consisted of consolidation and exudate similar to that described by Lamar and Meltzer<sup>5</sup> and Wollstein and Meltzer.<sup>6</sup> Filtrates from these consolidated lung tissues were without pronounced effect on normal rabbits when injected intratracheally.

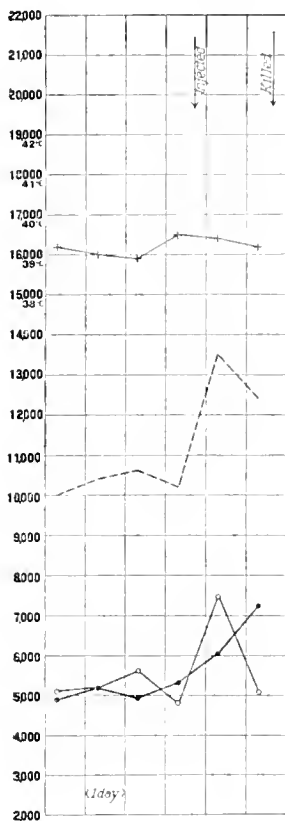
*B. Pfeifferi*.—The *B. Pfeifferi* used for inoculation was obtained from lungs of fatal cases of influenza, from exploratory thoracentesis, and from the sputum of cases of influenza. *B. Pfeifferi* were suspended in saline solution (12 to 60 billion per inoculation) and injected intratracheally. Three separate strains were injected. The passage of one of the strains through two rabbits in succession and of the other two through three rabbits was attempted. The secondary passages were made with ground and suspended lung tissues of the rabbits previously inoculated. The effect of the injections was to induce a leucocytosis which was generally of a polymorphonuclear type and was still present at the end of 48 hours when the animals were killed in order to observe the lung lesions (Text-figs. 1 to 3). Two of the strains induced no visible lung lesions; the third strain seemed to set up a pneumonic consolidation from which, however, not *B. Pfeifferi* but an atypical *Pneumococcus* Type II was isolated. In no instance was *B. Pfeifferi* recovered from the injected lungs.

The toxic extract described by Parker<sup>7</sup> was injected into the lungs of seven rabbits in doses of from 2 to 3 cc. In certain rabbits a polymorphonucleosis was set up, in others no blood changes were produced (Text-fig. 4). No visible pulmonary lesions resulted.

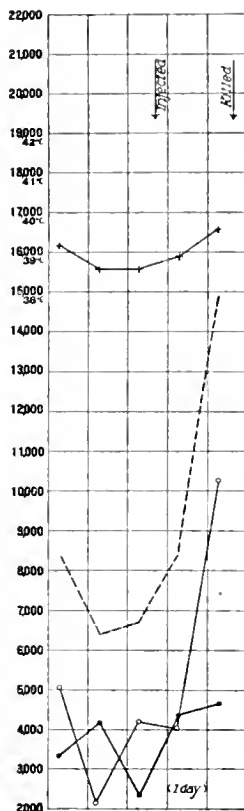
<sup>5</sup> Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

<sup>6</sup> Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 353.

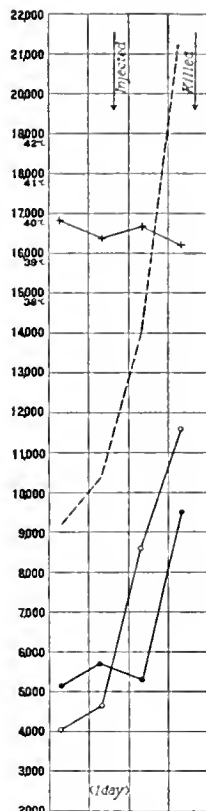
<sup>7</sup> Parker, J. T., *J. Immunol.*, 1919, iv, 331.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

+ Temperature  
 --- Total leucocytic count  
 ○ Mononuclears  
 ● Polymorphonuclears

TEXT-FIG. 1. Effect on the blood count and temperature of the intratracheal inoculation of *B. Pfeifferi*. First rabbit passage. The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy.

TEXT-FIG. 2. Effect on the blood count and temperature of the intratracheal inoculation of *B. Pfeifferi*. Second rabbit passage. The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy.

TEXT-FIG. 3. Effect on the blood count and temperature of the intratracheal inoculation of *B. Pfeifferi*. Third rabbit passage. The leucocytic rise, the polymorphonucleosis, and the variable temperature reaction are noteworthy.

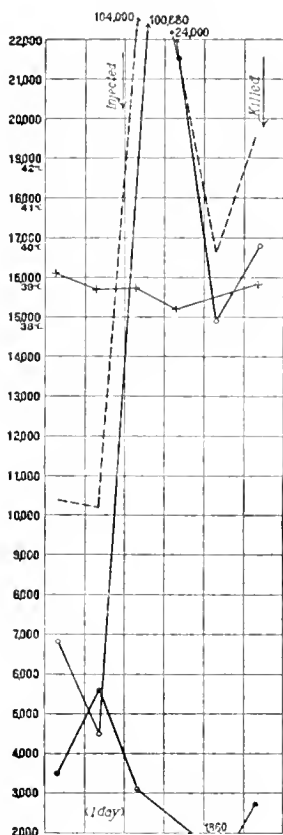
In other words, neither *B. pfeifferi* nor its toxic constituents when injected intratracheally into the lungs of rabbits induced lung or blood changes similar to those described for the influenzal agent, or set up a pneumonic consolidation, or led directly to the death of the animals. As a rule, a polymorphonuclear leucocytosis without distinctive lung lesions was produced.

*B. bronchisepticus*.—Small doses (one-fiftieth of the 18 hour growth on a standard agar slant) induced after several days a slowly developing polymorphonucleosis and leucocytosis with multiple abscesses and necrosis in the lungs; large doses (ten times as much) caused acute death.

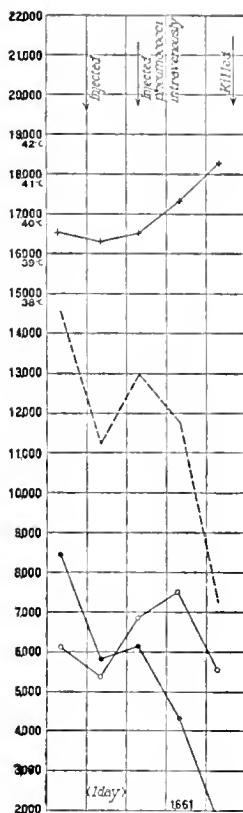
### *Experimental Reproduction of Concurrent Infections*

We now turned to the experiments on concurrent infection with the influenzal agent and ordinary bacteria. To review the main points reached or at issue we may state that pneumococci, *Bacillus pfeifferi* or its poison, and *Bacillus bronchisepticus*, in small doses, produce only transient effects when injected intratracheally in rabbits, and these effects differ essentially from the equally transient effects induced by the influenzal agent. It was next proposed to ascertain whether lungs already damaged by the influenzal agent would react differently to the bacterial inoculations. The method employed was to introduce the influenzal agent into the lungs and then to inject the bacteria either into the lungs or into the circulation. The latter route was adopted as a severer trial of the concomitant action, even though it may not be the route taken in the ordinary secondary infections of the lungs in man.

*Influenzal Agent Plus Pneumococci*.—May 31, 1919. A rabbit was injected intratracheally with 2.5 cc. of a suspension of glycerolated lung tissue corresponding to the fourteenth rabbit passage of the nasopharyngeal secretions from Patient 17, a case of influenza. June 1. Conjunctivitis; loss of weight (40 gm.); leucopenia (decrease of 2,075 cells); mononuclear depression (decrease of 612 cells). June 2. Injected intravenously with 5 million *Pneumococcus* Type IV, rabbit strain. June 3. Temperature 39.5°C.; total leucocytes increased 6,350 cells; mononuclears increased 4,904 cells. June 4. Temperature 40.5°C.; total leucocytes increased 2,875; mononuclears increased 54 cells. Died. The lungs showed consolidation of the right lower lobe, and hemorrhage, edema, and emphysema of other parts. From the lung and spleen *Pneumococcus* Type IV was isolated in culture.



TEXT-FIG. 4.



TEXT-FIG. 5.

TEXT-FIG. 4. Inoculation intratracheally of the poison of *B. Pfeifferi* as prepared by Parker's method. The marked polymorphonucleosis is shown.

TEXT-FIG. 5. Inoculation of a rabbit intratracheally with the **influenzal** agent followed in 24 hours by an intravenous injection of pneumococci. The depression in the leucocytic as well as in the mononuclear counts, and the **high** temperature are noteworthy. This animal was moribund when killed, and showed lobar consolidation, which yielded pneumococci, besides the **typical** lesions of the **influenzal** agent.

A second rabbit (Text-fig. 5) gave an identical result.

In order to control this experiment a third rabbit was injected intratracheally with 3 cc. of a suspension of normal rabbit lung tissue and then injected intravenously in the same manner as the preceding rabbit with 5 million pneumococci. Except for a transient leucocytosis 24 hours after the first injection, no effects were noted. The animal was killed 2 days after the injection of pneumococci. The lungs and other organs appeared normal.<sup>8</sup>

*Influenzal Agent Plus Streptococcus viridans or B. Pfeifferi.*—Experiments modelled precisely on those described for the pneumococcus were carried out with *B. Pfeifferi* and *Streptococcus viridans*. In each instance 24 hours after the intratracheal injection of the influenzal agent, a standard agar slant growth of *Streptococcus viridans* or one-half of such a growth of *B. Pfeifferi* suspended in saline solution was injected intravenously. Preceding the intravenous injections the characteristic blood changes due to the agent were present. The animals injected with the streptococcus usually succumbed on the 3rd day and showed a massive consolidation of the lungs with fibrinous pleuritis. Cultures from the lung tissue yielded an abundant growth of *Streptococcus viridans*. A control rabbit given normal lung tissue suspension intratracheally and the streptococci intravenously was killed after 3 days, and at autopsy failed to show lung changes. No growth of the streptococcus was obtained from the lung tissue.<sup>9</sup>

The combined influenzal agent and *B. Pfeifferi* injections set up a bronchopneumonic consolidation of one or more pulmonary lobes, and a hemorrhagic edema of the remainder of the lung. The cultures yielded abundant *B. Pfeifferi*. A control rabbit injected intratracheally with normal lung suspension and intravenously with *B. Pfeifferi* showed a transient polymorphonuclear leucocytosis. The lungs were not visibly altered.

These severe tests showed the conditions under which concurrent infections arose experimentally in the rabbit. A method was next employed in rabbits which imitated more closely the manner of development of secondary infections in influenza in man.

<sup>8</sup> Larger or fatal doses of virulent pneumococci injected intravenously resulted in a generalized pneumococcic septicemia in rabbits inoculated intratracheally with normal rabbit tissue; all organs, including the lungs, were congested and yielded the pneumococcus on culture. The rabbits inoculated intratracheally with the influenzal agent, however, in similar procedures showed definite localization of the bacterial infection in the lungs—fibrinous and exudative pleuritis, and consolidation—besides the involvement of other organs.

<sup>9</sup> In the case of *Streptococcus viridans* an experiment was also made in which these bacteria were injected intravenously 4 hours before the intratracheal inoculation of the influenzal agent. The results were similar.

*Influenzal Agent Injected Intratracheally with Pneumococcus Type IV, Streptococcus viridans, or B. Pfeifferi.*—A series of rabbits was injected intratracheally with the influenzal agent together with small non-pathogenic doses of Pneumococcus Type IV, *Streptococcus viridans*, or *B. Pfeifferi*. These experiments, properly controlled, showed that effects were produced similar to those obtained when these bacteria were injected intravenously; namely, more or less extensive consolidation, either lobar or bronchial, and from the affected lungs the same kind of microorganisms which were inoculated were recovered in pure culture, while the remainder of the lungs showed the hemorrhagic edema with emphysema characteristic of the influenzal agent (Figs. 1 and 2).

The experiments just given have an important bearing on the subject of this study since they show that the intratracheal injection of the influenzal agent in rabbits exerts an influence on the pulmonary structures of these animals of a nature to encourage the invasion of the lung and the subsequent multiplication there, with lethal outcome, of such bacteria as the pneumococcus, streptococcus, and *Bacillus Pfeifferi*, which otherwise, in the doses employed, are without marked effect. The control experiments show that the injection of normal rabbit lung exerts no such predisposing influence. While the experiments are perhaps not an exact reproduction of the conditions occurring in man in secondary pneumonia following influenza they bear directly on these conditions.

#### CONCLUSIONS.

1. Concurrent infections in the experiments described may be regarded as of accidental nature and are not causally related to the typical effects induced in rabbits by a material wholly free from ordinary bacteria.

2. The influenzal agent exerts an effect on the pulmonary tissue which encourages the invasion of the lung and subsequent multiplication there of ordinary bacteria, such as the pneumococcus, streptococcus, and *Bacillus Pfeifferi*.

3. A similarity is believed to exist between the conditions under which concurrent infections arose in the inoculated rabbits and those which seem to favor the occurrence of concurrent infections during epidemic influenza in man. In no instance did death occur in the rabbits as a result of the uncomplicated effects of the influenzal

agent alone. When death occurred in any of the inoculated animals concurrent infection of the lungs by ordinary bacteria was present. The microorganisms most commonly met with under these conditions were *Pneumococcus* Type IV and atypical Type II, streptococci, and hemoglobinophilic bacilli. Other kinds were encountered less often.

#### EXPLANATION OF PLATE 35.

FIG. 1. The influenzal agent with small numbers of *Pneumococcus* Type IV was inoculated intratracheally in a rabbit. The right lung shows the typical effects of the influenzal agent; it is voluminous, emphysematous, edematous, and hemorrhagic. The left lung shows massive lobar consolidation (red hepatization) and yielded on culture a profuse growth of the pneumococci. Natural size.

FIG. 2. The influenzal agent with *B. Pfeifferi*, both ordinarily without effect by themselves, was inoculated intratracheally in a rabbit. The left lung shows the typical effects of the influenzal agent; it is voluminous, emphysematous, edematous, and hemorrhagic. The right lung shows patchy consolidation (bronchopneumonia) and yielded on culture *B. Pfeifferi*. Natural size.

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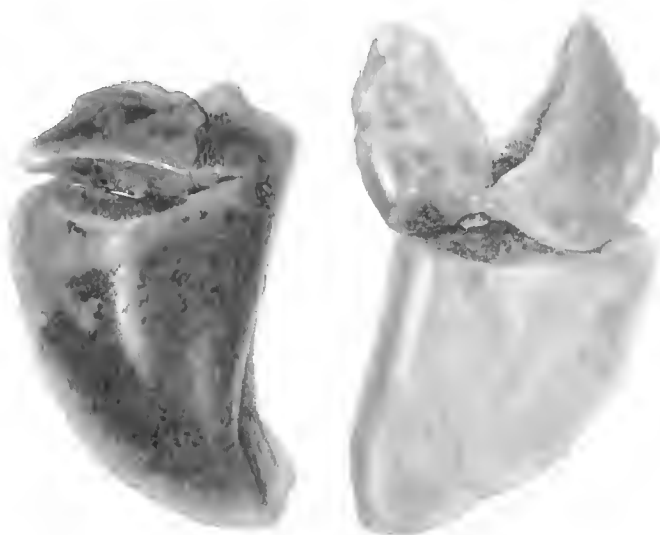


FIG. 1.



FIG. 2.



## STUDIES ON MEASLES.

### I. SUSCEPTIBILITY OF MONKEYS TO THE VIRUS OF MEASLES.

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PLATE 36.

(Received for publication, November 5, 1920.)

#### INTRODUCTION.

Measles occupies a prominent position among the acute infectious diseases that lead to severe and not infrequently fatal secondary infections of the respiratory tract. Efforts to control this serious effect of measles are at present directed almost wholly toward the prevention of the secondary infections by means of isolation and careful treatment of the patient, and while they have met with a certain degree of success, the results obtained are still far from satisfactory. A more hopeful solution of the problem would appear to lie in the prevention of measles itself.

Long experience has shown that the prevention of measles by strict quarantine is largely inadequate because of the contagiousness of the disease during the prodromal period. There would appear to be, however, more than a theoretical possibility that prevention may be accomplished by protective inoculation. This will obviously require isolation of the virus of measles, or at least the development of a method of handling the virus in pure form, whether it is actually identified under the microscope and in the culture tube or not. Much work has already been done in this direction, both by culture study and by attempted transmission of the virus to animals. The former method has not yet yielded positive results. Whether the transmission of the virus to animals has been successfully accomplished has remained an open question because of the conflicting results obtained by different investigators and because of the somewhat indefinite and limited criteria that have been relied upon as evidence

of a positive transmission. In undertaking a study of measles it has seemed desirable, therefore, to determine at the outset whether more conclusive evidence concerning the susceptibility of animals to the virus of the disease might not be obtained.

This paper presents the results of experiments so far conducted on the transmission of measles from man to monkeys and the passage of the infection from monkey to monkey. The symptomatology and pathology of the reaction produced will be presented in greater detail in a subsequent paper.<sup>1</sup>

#### LITERATURE.

The more recent experiments upon the transmission of measles to monkeys date from the work of Anderson and Goldberger<sup>2</sup> and are largely based upon Hektoen's<sup>3</sup> earlier report of having successfully transmitted measles from man to man by the subcutaneous injection of ascites broth blood cultures from patients in the early eruptive stage of the disease. The former authors claim to have transmitted the virus of measles to monkeys in a considerable number of instances by the use of whole defibrinated blood, blood serum, and washed corpuscles from cases of measles in the preeruptive or early eruptive stages of the disease. Intraperitoneal, intracerebral, intravenous, and subcutaneous routes of inoculation were employed with apparently equal success. Transmission was also accomplished by subcutaneous injection of nasopharyngeal secretions collected in the prodromal and early eruptive stages, and further transmission from monkey to monkey was carried through six passages. The evidence of a positive transmission consisted in a moderate febrile reaction of inconstant character coming on after an incubation period varying from 6 to 21 days, accompanied in some instances by an erythematous or maculopapular rash of variable character with or without symptoms of rhinitis. No mention is made of Koplik spots, of blood counts, or of histological examination of the skin or mucous membranes. It seems probable that Anderson and Goldberger succeeded in transmitting the virus of measles to monkeys in certain of their experiments, but the evidence which they present is not entirely conclusive.

Nicolle and Conseil<sup>4</sup> using similar methods have also reported the successful transmission of the virus of measles from man to monkey and its further passage

<sup>1</sup> Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 413.

<sup>2</sup> Anderson, J. F., and Goldberger, J., *Pub. Health Rep., U. S. P. H.*, 1911, xxvi, 847, 887; *J. Am. Med. Assn.*, 1911, lvii, 113. Goldberger, J., and Anderson, J. F., *J. Am. Med. Assn.*, 1911, lvii, 476. Anderson, J. F., and Goldberger, J., *J. Am. Med. Assn.*, 1911, lvii, 1612.

<sup>3</sup> Hektoen, L., *J. Infect. Dis.*, 1905, ii, 238.

<sup>4</sup> Nicolle, C., and Conseil, E., *Compt. rend. Acad.*, 1911, cliii, 1522; *Compt. rend. Soc. biol.*, 1920, lxxxiii, 56.

from monkey to monkey. Except for an elevation of temperature after an incubation period of 8 to 11 days no symptoms of measles are mentioned as having been observed in the inoculated monkeys.

Hektoen and Eggers,<sup>5</sup> Tunncliffe,<sup>6</sup> and Lucas and Prizer<sup>7</sup> have studied the leucocyte reaction in monkeys inoculated with blood from measles patients and have shown that a fall in the total leucocyte count takes place. These authors, however, present little definite evidence that the monkeys were infected with the virus of measles.

Jurgelunas<sup>8</sup> inoculated three monkeys with defibrinated blood and five monkeys with secretions of the respiratory tract from cases of measles. He also exposed two monkeys for 5 days to active cases of measles in a hospital ward. None of the animals showed any evidence of infection with the virus of measles.

Sellards and Wentworth<sup>9</sup> and Sellards<sup>10</sup> have recently carried out a series of inoculation experiments in both monkeys and man. Five monkeys were intensively inoculated with large amounts of blood from preeruptive and early eruptive cases of measles. The results were negative. Eight susceptible human volunteers were inoculated with blood from cases of measles in the prodromal or early eruptive stages. None of the men showed any evidence of measles.

Although according to the literature, as Sellards has pointed out, most of the important symptoms of measles have been described in inoculated monkeys, it is striking that no single investigator has obtained all the features in any one animal or even in a series of animals, and that no single symptom has appeared with constancy. The periods of incubation vary widely, the temperature reactions are inconstant, Koplik spots have been noted only by Lucas and Prizer, the rashes described are variable in character and frequently lacking, and there is disagreement in regard to the character of the leucocyte reaction.

#### EXPERIMENTAL.

In attempting to determine the susceptibility of monkeys to the virus of measles it seemed desirable at the outset to utilize a method that presumably would afford the optimum opportunity for successful transmission; namely, the use of a comparatively large amount of material believed to contain the virus of measles and the inoculation

<sup>5</sup> Hektoen, L., and Eggers, H. E., *J. Am. Med. Assn.*, 1911, lvii, 1833.

<sup>6</sup> Tunncliffe, R., *J. Infect. Dis.*, 1912, xi, 474.

<sup>7</sup> Lucas, W. P., and Prizer, E. L., *J. Med. Research*, 1912-13, xxvi, 181.

<sup>8</sup> Jurgelunas, A., *Centr. Bakt., Ite Abt., Orig.*, 1914, lxxii, 483.

<sup>9</sup> Sellards, A. W., and Wentworth, J. A., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 57.

<sup>10</sup> Sellards, A. W., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 257, 311.

of this material by the natural path of infection. This method might be expected to afford not only the best opportunity for successful transmission, but also a greater probability that the reaction of the animal to the virus would more closely conform to the course of measles as it occurs in man. Clinical observation has indicated beyond reasonable doubt that the virus of measles is abundantly present in the secretions of the respiratory tract during the pre-eruptive and early eruptive stages of the disease and that the respiratory mucous membrane is the natural path of entry of the virus. The method used in the preliminary experiments, therefore, consisted in inoculation of the mucous membranes of the respiratory tract with unfiltered nasopharyngeal secretions of patients in the early stages of measles. The secretions were collected by irrigation of the nasopharynx with 20 to 40 cc. of sterile 0.85 per cent salt solution. Monkeys were inoculated with 5 to 10 cc. of the nasopharyngeal washings by intratracheal injection in order to facilitate retention by the animal of as much of the material as possible. When as much as 5 to 10 cc. was injected a small amount was commonly regurgitated and spread itself over the mucous membranes of the buccal and nasal cavities.

It is appreciated that this method is open to criticism on the ground that many other organisms beside the virus of measles were inevitably introduced into the trachea. It was felt, however, that in most instances these organisms, being largely saprophytic, would be promptly disposed of by the normal respiratory mucous membrane and would not interfere with the ultimate result of the experiment. It furthermore seemed probable that other methods free from the foregoing objection could more satisfactorily be carried out after the susceptibility of the monkey to the supposed virus of measles had been demonstrated and the criteria necessary to establish a positive transmission had been learned. This objection has been met in subsequent experiments by the use of filtered nasopharyngeal washings and of blood shown to be free from ordinary bacteria by culture tests.

The methods of study after inoculation consisted in daily observation of symptoms and inspection of the skin, conjunctivæ, and mucous membranes of the mouth, in the recording of morning and evening temperature (rectal), and daily counts of the white blood corpuscles.

Blood cultures were made at irregular intervals in a variety of media (aerobic and anaerobic) both during the incubation period and during the disease in order to exclude as far as possible the presence of possible intercurrent infections resulting from the introduction of extraneous organisms into the trachea, or occurring independently from other sources. Finally, small sections of skin were excised<sup>11</sup> in nearly all animals during the course of the exanthem in order to determine the histological character of the lesion. All animals were observed for a period of at least 3 weeks after inoculation before being discarded as negative. The evidence for a positive transmission has depended, as in the clinical diagnosis of measles, upon the development of the characteristic symptoms and lesions of the disease, the temperature and leucocyte counts having been recorded merely as additional data and not as evidence of a successful inoculation.

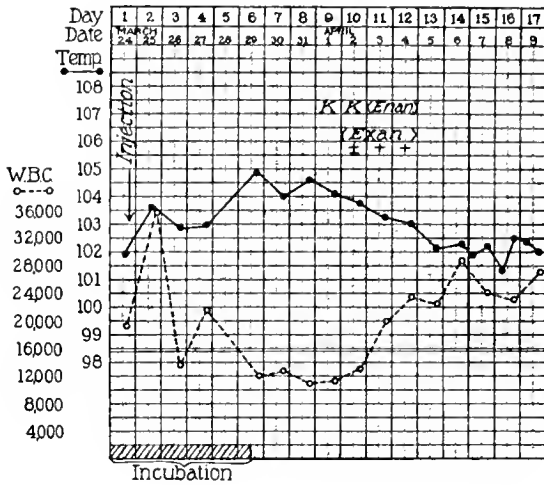
*Direct Transmission of the Virus of Measles from Man to Monkeys.*

Of ten monkeys inoculated with nasopharyngeal washings from seven patients with measles, eight after an incubation period of 6 to 10 days developed symptoms closely resembling those of measles in man. Of the two monkeys which failed to develop symptoms of measles, one contracted a Pneumococcus Type IV pneumonia with a severe pneumococcus septicemia 24 hours after inoculation and died on the 8th day, while the other failed to show sufficiently definite symptoms to warrant a positive diagnosis and was discarded as negative after 24 days observation. The details of the experiments in which a positive transmission was obtained follow.

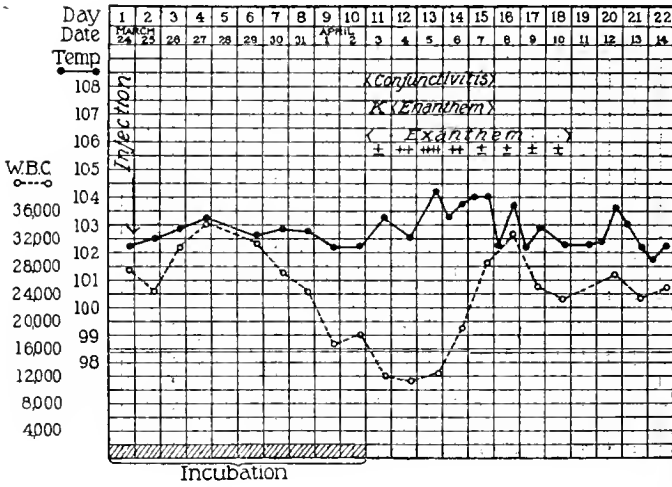
In the first two experiments monkeys were inoculated intratracheally with unfiltered nasopharyngeal washings. In the first instance the secretions were collected from the patient about 1 hour after the first appearance of the exanthem, in the second 6 days before the exanthem appeared.

*Experiment 1.*—Monkey 2 (Text-fig. 1, a); *Macacus rhesus*. Mar. 24, 1920. Intratracheal injection of 10 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 1, about 1 hour after the first appearance of the exanthem. Cultures of the washings showed *Micrococcus catarrhalis*, *Streptococcus viridans*, *B. influenzae*, *Staphylococcus pyogenes aureus*, and diphtheroid bacilli.

<sup>11</sup> This was always done under anesthesia.



a



b

TEXT-FIG. 1, a and b. Observations on monkeys inoculated with unfiltered nasopharyngeal washings from patients with measles. (a) Monkey 2, Case 1. (b) Monkey 3, Case 2.



The monkey remained well and active until the 6th day when the temperature rose to 104.7° F. and remained elevated for 5 days. The leucocyte count fell on the 6th day and remained low for 5 days. On the 9th day the animal became less active; there was photophobia, and a small, bright red macule appeared on the mucous membrane of the left lower gum. Blood culture showed no growth. On the 10th day drowsiness was quite marked; two more discrete, erythematous spots were present on the labial mucous membrane and a rash consisting of small, discrete, red maculopapules which faded out on pressure appeared on the forehead, cheeks, and neck. On the 11th day the exanthem was more prominent but had not spread to other parts of the skin. The small hyperemic spots on the labial mucous membrane were more numerous but not coalescent. On the 12th day the enanthem was fading and the exanthem was less marked; by the 13th it had disappeared without definite pigmentation or desquamation and the animal again appeared well and active.

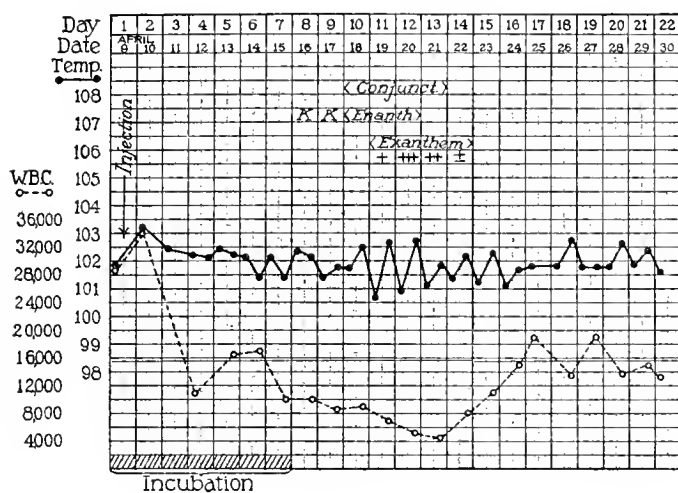
*Experiment 2.*—Monkey 3 (Text-fig. 1, b); *Macacus rhesus*. Mar. 24, 1920. Intratracheal injection of 10 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 2, 6 days before the appearance of the exanthem. Cultures of the washings showed *Streptococcus viridans*, *B. influenzae*, *Staphylococcus pyogenes albus*, *Staphylococcus pyogenes aureus*, and diphtheroid bacilli. The animal remained well and active for 10 days. On the 11th day it appeared quiet; the conjunctivæ were markedly congested; a small, bright red macule appeared on the mucous membrane of the upper lip, and in the afternoon a few, small, red, discrete maculopapules appeared on the forehead and cheeks. On the 12th day the conjunctivitis was more marked; there was a moderate, erythematous, granular rash on the mucous membrane of the lips and an abundant, bright red, maculopapular exanthem over the entire face, in places nearly confluent. On the 13th day the animal was weak and drowsy; the exanthem had spread to the flexor surfaces of the arms, forearms, and thighs. On the 14th day the rash had begun to fade. During the 4 following days it gradually disappeared; first from the arms and legs and finally from the face. By the 19th day it had entirely gone, leaving only a yellowish brown pigmentation which lasted for several days and then cleared up. No desquamation was noted. The animal showed a well defined febrile reaction from the 11th to 16th days coincident with the other symptoms. The leucocyte count was low from the 9th to 14th days. On the 3rd day of the exanthem small pieces of skin were excised from the face, left arm, and left thigh. All show the characteristic histology of the measles exanthem, consisting of a proliferative and exudative reaction about the capillaries in the corium. The endothelial cells of the capillary walls are swollen, and numerous endothelial leucocytes are present about the capillaries. A moderate number of these cells are in mitosis, indicating an active multiplication. A few eosinophils, polymorphonuclear leucocytes, and lymphocytes are also present in the pericapillary exudate. There are a few small foci of exudative and degenerative changes in the epidermis. At these points the epithelial cells are swollen and vacuolated, and occasionally show evi-

dence of necrosis. Similar changes are seen in small groups of cells in the hair sheaths and sebaceous glands that lie adjacent to the capillary lesions.

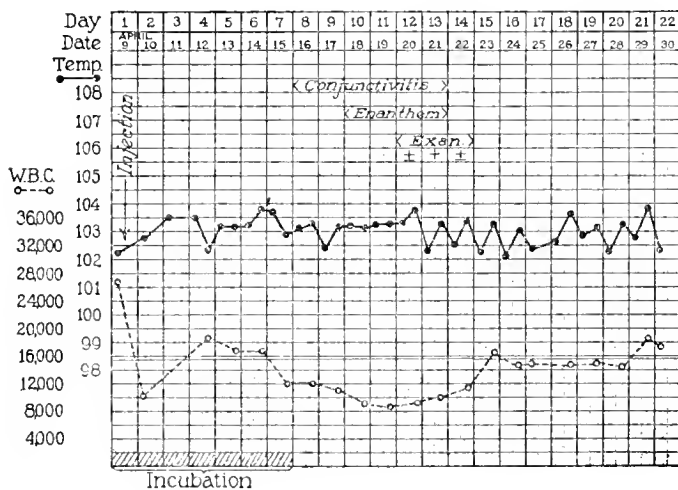
In the next experiment two monkeys were inoculated intratracheally with the pooled nasopharyngeal washings from two patients in the prodromal stage of measles 3 days before the appearance of the exanthem. One monkey received unfiltered washings, the other a portion of the same washings after filtration through a new Berkefeld N filter.

*Experiment 3.*—Monkey 5 (Text-fig. 2, a); *Macacus rhesus*. Apr. 9, 1920. Intratracheal injection of 10 cc. of pooled unfiltered nasopharyngeal washings collected from measles patients, Cases 3 and 4, 3 days before the appearance of the exanthem. Cultures of the washings showed *Staphylococcus pyogenes albus*, *Micrococcus catarrhalis*, *Streptococcus viridans*, and diphtheroid bacilli. The monkey remained free from symptoms for 6 days. On the afternoon of the 7th day it appeared quiet and drowsy and shivered at times. On the 8th day two small, hyperemic spots appeared on the mucous membrane of the upper lip. On the 10th day the conjunctivæ were slightly congested and a red, punctate, granular rash was present on the labial mucous membrane. On the 11th day a well defined rash consisting of small, discrete, red maculopapules had appeared on the forehead, cheeks, sides of neck, upper chest, lower abdomen, and inside of thighs. The conjunctivitis remained the same; the enanthem was more marked. On the 12th day the exanthem was more abundant; the individual maculopapules were larger, and fresh spots had appeared over the shoulders. The enanthem was fading. The exanthem rapidly faded on the 2 following days. By the 15th day the animal had completely recovered. No pigmentation or desquamation was noted. There was a well defined leucopenia from the 7th to 15th days. Blood cultures made on the 4th and 7th days showed no growth. Sections of skin removed from the face and thigh on the 2nd day of the exanthem show the characteristic histology of measles.

Monkey 6 (Text-fig. 2, b); *Macacus rhesus*. Apr. 9, 1920. Intratracheal injection of 10 cc. of pooled, filtered (Berkefeld N, 20 minutes, 640 mm. vacuum) nasopharyngeal washings collected from measles patients, Cases 3 and 4, 3 days before the appearance of the exanthem. Aerobic and anaerobic cultures of the filtrate showed no growth during 2 weeks incubation except in one of four anaerobic tissue ascites fluid tubes in which a small Gram-negative bacillus, presumably a contaminant, appeared. The monkey remained well and active for 7 days. On the 8th day it showed a moderate conjunctivitis which had increased on the following day. On the 10th day the mucous membranes were congested and showed a moderate, hyperemic, punctate rash on the lips. On the 12th day a few, small, discrete, red maculopapules came out on the forehead, left cheek, and front of the neck. On the 13th day there was a well marked, though sparse,



a



b

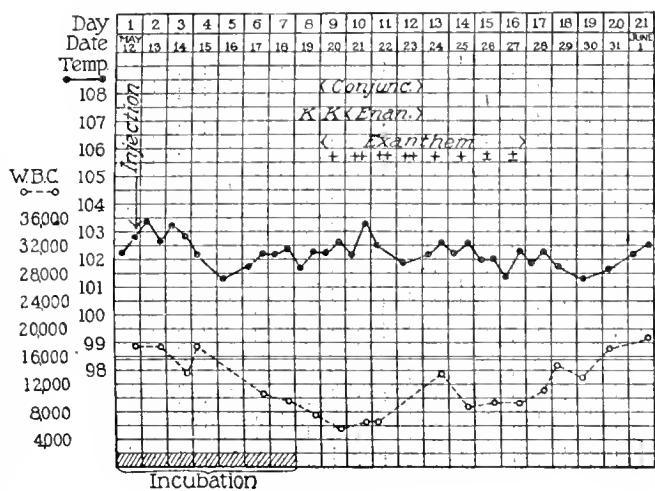
TEXT-FIG. 2, *a* and *b*. Observations on monkeys inoculated with pooled nasopharyngeal washings from measles patients, Cases 3 and 4. (*a*) Monkey 5, unfiltered washings. (*b*) Monkey 6, filtered washings.

exanthem on the forehead, cheeks, and neck, and fresh maculopapules had appeared on the lower abdomen. On the 14th day the exanthem had disappeared and the exanthem had begun to fade. On the 15th day it had disappeared without desquamation; the animal appeared well and active. There was no febrile reaction. The leucocyte count was low from the 7th to 14th days. Blood cultures on the 2nd, 4th, and 7th days showed no growth. A piece of skin excised from the forehead on the 2nd day of the exanthem shows the typical histological picture of measles. This animal was subsequently reinoculated on June 8, 1920, with material containing another strain of the virus of measles. It failed to react, the control coming down on the 7th day with characteristic symptoms of measles.

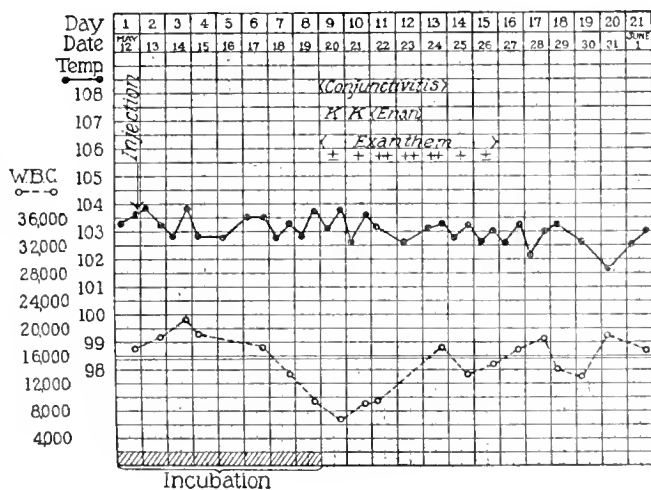
In the following experiment four monkeys were inoculated with different portions of the nasopharyngeal washings collected from a patient with measles about 22 hours after the appearance of the exanthem. One monkey was inoculated on the mucous membranes of the nose and throat with unfiltered washings, one intratracheally with filtered (Berkefeld N) washings, and two intratracheally with unfiltered washings. All developed the characteristic symptoms of measles after the customary incubation period. The protocols follow.

*Experiment 4.*—Monkey 8 (Text-fig. 3, a); *Macacus rhesus*. May 12, 1920. Inoculated on mucous membranes of nose and throat with 5 cc. of unfiltered nasopharyngeal washings from measles patient, Case 5. Cultures of the washings showed *Staphylococcus pyogenes albus*, *Streptococcus viridans*, and diphtheroid bacilli. The animal remained well for 7 days. On the 8th day it appeared quiet; the tongue was coated and its papillæ were prominent; two small, bright red spots appeared on the mucous membrane of the upper lip. On the 9th day it was quiet and limp; the conjunctivæ were injected; the mucous membranes of the cheeks were congested and a third hyperemic spot was present on the upper lip. A red, maculopapular rash appeared on the inner and posterior sides of the thighs and over the perineum. On the 10th day the exanthem was more intense and had spread to the anterior surfaces of the thighs and lower abdomen. On the 11th day the exanthem on the thighs had begun to fade. Fresh maculopapules were present on the abdomen and on the left cheek. The conjunctivitis and exanthem were less marked. By the 14th day the animal appeared well and the exanthem had nearly faded, leaving a yellowish brown pigmentation. There was a well marked leucopenia coincident with the foregoing symptoms but no definite febrile reaction. A section of skin removed from the thigh on the 2nd day of the exanthem shows the characteristic histological picture of measles.

Monkey 9 (Text-fig. 3, b); *Macacus rhesus*. May 12, 1920. Intratracheal injection of 4.5 cc. of filtered (Berkefeld N, 5 minutes, 600 mm. vacuum) nasopharyngeal washings from measles patient, Case 5. Aerobic and anaerobic cul-



a



b

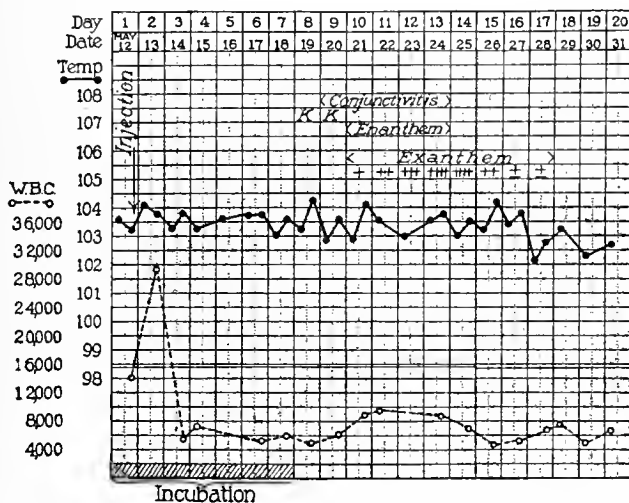
TEXT-FIG. 3, *a* and *b*. Observations on monkeys inoculated with nasopharyngeal washings from measles patient, Case 5. (*a*) Monkey 8, unfiltered washings. (*b*) Monkey 9, filtered washings.

tures of the filtrate showed no growth after 2 weeks incubation. The animal remained well and active for 8 days. On the 9th day it appeared quiet; the conjunctivæ were moderately injected. On the mucous membrane of the upper lip there was a small, red, granular spot and on that of the left cheek there were three slightly elevated, hyperemic spots with bluish white centers presenting the typical appearance of Koplik spots. A few red maculopapules developed on the skin about the left corner of the mouth. On the 10th day the condition was the same, except for an additional spot on the mucous membrane of the lower lip and fresh maculopapules on the left cheek. On the 11th day there was a well developed, red, punctate, granular enanthem on the mucous membrane of the lips; the exanthem had spread to the forehead, cheeks, and nose. On the 13th day the enanthem had cleared up; the conjunctivitis was less marked; the exanthem on the face was fading. A red, maculopapular rash had appeared on the thighs. During the 2 following days the exanthem faded with a fine, branny desquamation, and the animal again became lively. There was no significant febrile reaction, but a well defined leucopenia occurred from the 8th to 11th days. This animal was reinoculated on June 8, 1920, with material containing the same strain of measles virus after it had been passed through three monkeys. It failed to react, while the control after 6 days incubation period developed the characteristic symptoms of measles.

Monkey 10 (Text-fig. 4); *Macacus rhesus*. May 12, 1920. Intratracheal injection of 5 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 5. The monkey remained well for 7 days. On the 8th day it was listless and drowsy, shivering slightly; a small hyperemic spot was present on the mucous membrane of the lower lip. The tongue was coated and its papillæ were prominent. On the 9th day the conjunctivæ were inflamed; there was a faint erythematous blush over the face and two fresh red macules were present on the mucous membrane of the upper lip. On the 10th day there was a well developed, red, granular enanthem on the labial mucous membrane; a few, small, discrete, red maculopapules appeared on the left cheek and on the upper arms. By the 13th day the exanthem had reached its height. It consisted of numerous, red maculopapules, 2 to 5 mm. in diameter, irregularly scattered over the face, neck, chest, upper arms, abdomen, and thighs (Fig. 1). On the 14th day the conjunctivæ and mucous membranes appeared normal again; the exanthem on the face and neck was yellowish red and showed a fine desquamation. During the 3 following days the exanthem gradually faded with well marked branny desquamation. By the 18th day the animal again appeared well and active. A section of skin removed from the upper arm on the 1st day of the exanthem shows the typical lesion of measles.

Monkey 11 (Text-fig. 5, a); *Macacus rhesus*. May 12, 1920. Intratracheal injection of 5 cc. of unfiltered nasopharyngeal washings collected from measles patient, Case 5. The animal remained well until the afternoon of the 6th day when its temperature rose from 103.5° to 104.7° F. and it appeared quiet. On the

7th day it appeared listless and drowsy; the temperature remained elevated. Blood culture showed no growth. On the 8th day there was diarrhea; the conjunctivæ were inflamed and there was increased lacrimation; the mucous membrane of the lips was congested and showed two characteristic hyperemic spots; the temperature rose to 105.8° F. On the 9th day a well defined exanthem consisting of small, discrete, red maculopapules appeared about the eyes, on the nose, right cheek, chin, and about the corners of the mouth. The animal was killed for passage of the virus.



TEXT-FIG. 4. Observations on Monkey 10 inoculated with unfiltered nasopharyngeal washings from measles patient, Case 5.

*Autopsy.*—Grossly negative. Histological sections of the skin, labial mucous membrane, and tongue show the typical lesions of measles. Cultures of the heart's blood showed no growth.

The foregoing experiments have shown that monkeys injected intratracheally with nasopharyngeal washings from cases of measles in the preeruptive and early eruptive stages of the disease react after an incubation period varying from 6 to 10 days with a constant and characteristic group of symptoms closely resembling those of measles in man. These symptoms in brief are as follows: (1) onset with listlessness and drowsiness after a definite incubation period during which the animal appears well; (2) catarrhal conjunctivitis; (3) a well defined and characteristic exanthem usually confined to the labial

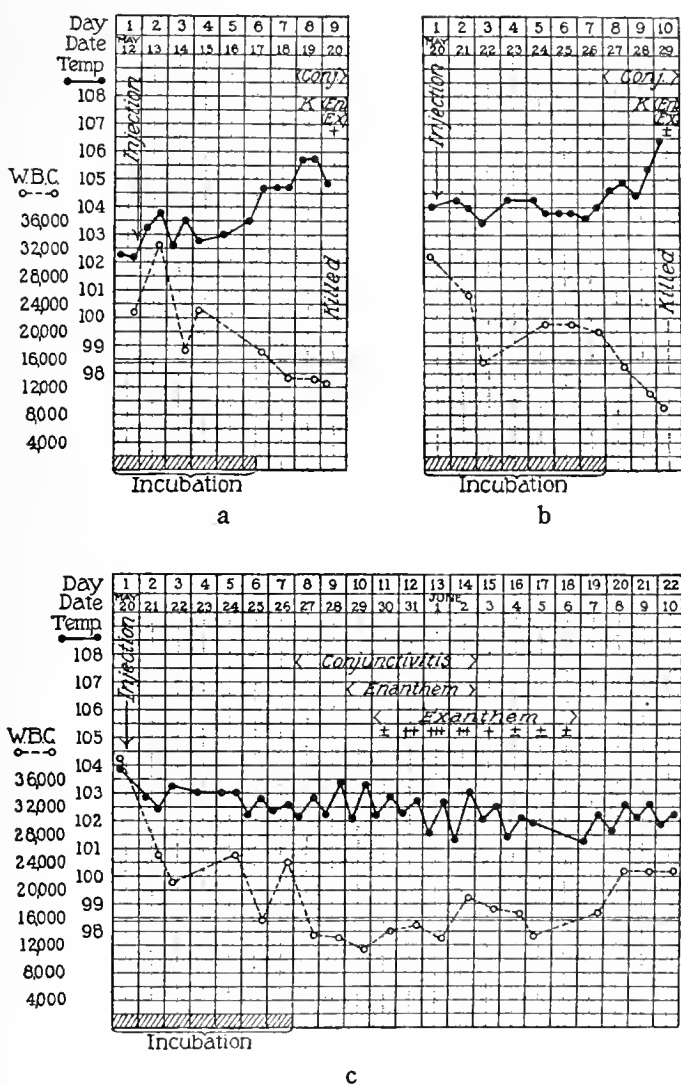
mucous membrane; (4) a definite exanthem consisting of discrete, red maculopapules constant in character though somewhat variable in extent and duration and comparable histologically with the exanthem of measles; (5) leucopenia coincident with the foregoing symptoms; (6) prompt and complete recovery after an illness of 7 to 10 days duration. Variations that have occurred in this group of symptoms are ones of degree in the severity of the reaction rather than in character. In only two respects has the reaction shown any significant variation from the symptoms of measles in man; namely, in the inconstant occurrence of a definite elevation of temperature and in the entire absence of symptoms of rhinitis and bronchitis.

The regularity with which this group of symptoms has developed and the close resemblance of the symptoms to the symptoms of measles are presumptive evidence that the reaction is due to the virus of measles. That the reaction is not due to ordinary organisms of the mouth flora inevitably present in unfiltered nasopharyngeal washings is shown by the occurrence of the same reaction following the inoculation of washings freed from ordinary bacteria by filtration. This, of course, does not exclude the possibility that the reaction might be caused by filterable toxic substances contained in the nasopharyngeal secretions of measles patients rather than by the living virus of measles. This possibility, however, has been excluded by the successful transmission of the infection through a considerable series of monkeys as described below.

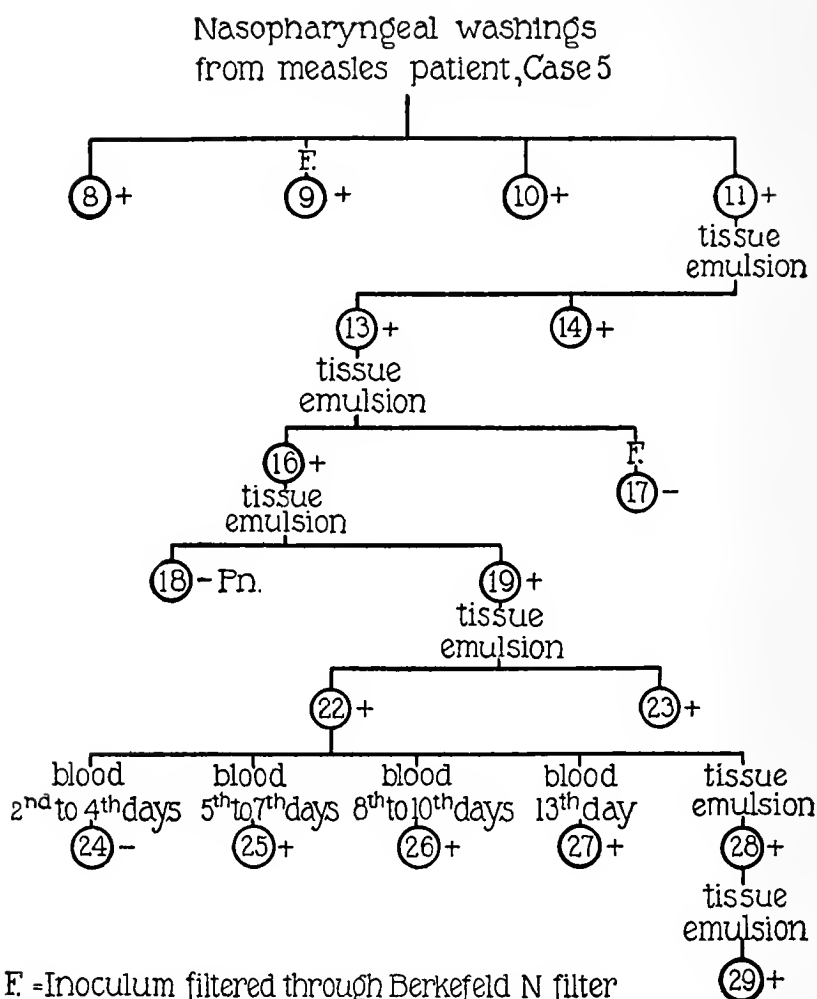
*Transmission of Measles from Monkey to Monkey by Intratracheal Injection of Tissue Emulsions.*

The characteristic reaction which follows the inoculation of monkeys with nasopharyngeal secretions of measles patients has been successfully carried through six passages (Text-fig. 6), by the intratracheal injection of salt solution tissue emulsions prepared from animals killed shortly after the appearance of the exanthem. The principal tissues used were skin and buccal mucous membrane. In some cases bits of spleen, liver, and lung were also employed. In three instances attempted passage failed, presumably due in two to absence of the virus of measles in the material inoculated, in one to the development of a fatal intercurrent infection. The details of the experiment follow.





TEXT-FIG. 5, *a* to *c*. Transmission of measles virus, strain from Case 5, from monkey to monkey, by means of tissue emulsions. (*a*) Monkey 11, inoculated with nasopharyngeal washings from measles patient, Case 5. (*b*) Monkey 13, first passage. (*c*) Monkey 14, first passage, duplicate of Monkey 13.



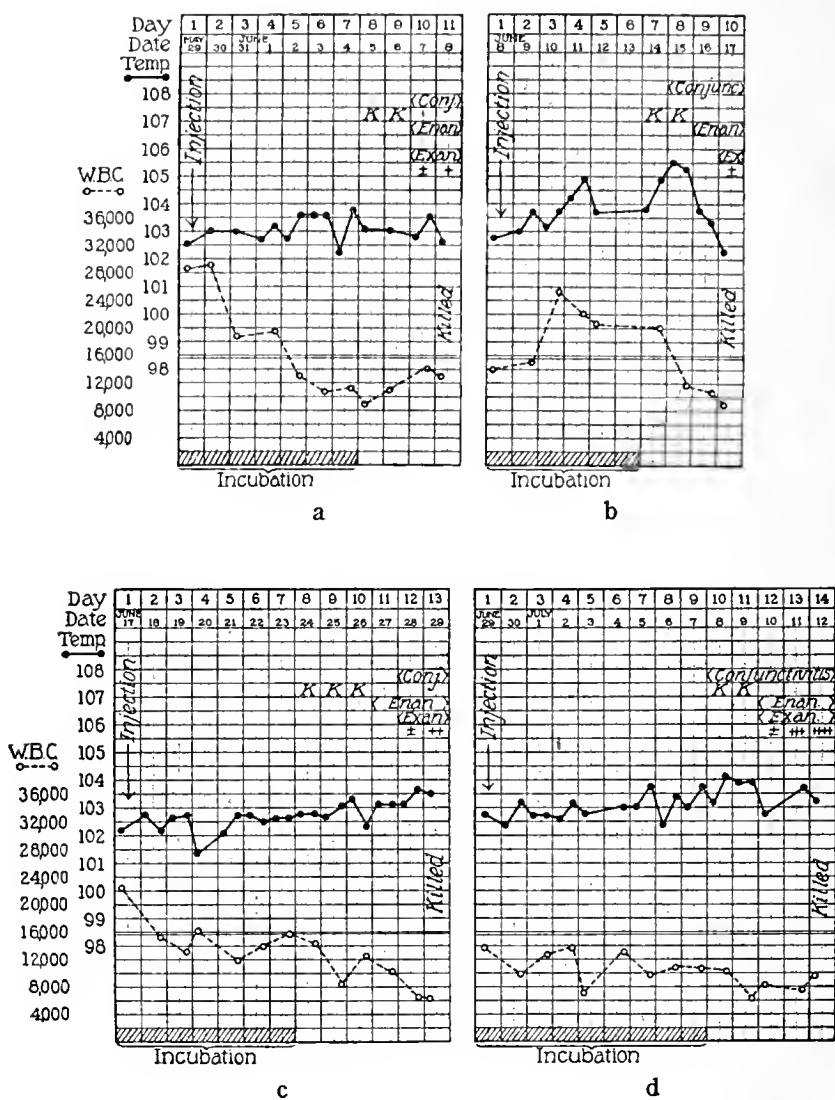
TEXT-FIG. 6. Transmission of measles virus, strain from Case 5, through six animal passages. Monkeys 8 and 23 were inoculated on the nasal and buccal mucous membrane, Monkeys 24 to 27 intravenously, the other monkeys intra-tracheally. 2nd to 4th days, etc., in Monkeys 24 to 27 indicate the days after inoculation of Monkey 22 on which blood was withdrawn from Monkey 22.

*Experiment 5. First Passage.*—Monkey 13 (Text-fig. 5, b); *Macacus rhesus*. May 20, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, liver, and lung) from Monkey 11. Monkey 11 (Text-fig. 5, a) was killed on the 1st day of the exanthem, the 9th day after inoculation. The hair was shaved from the face, front of chest, abdomen, and thighs, and the skin thoroughly scrubbed with soap and water. The skin was removed from these areas under aseptic precautions, cut into small bits with sterile scissors and ground with small pieces of spleen, liver, and lung in a sterile mortar with sterile sand and 40 cc. of 0.85 per cent salt solution, the whole procedure requiring about 1 hour. The supernatant fluid of this emulsion was pipetted off and used for inoculation without filtration or centrifugation. Cultures of the fluid on blood agar plates showed a few colonies of *Staphylococcus albus*. Monkey 13 remained well and active for 7 days. On the 8th day the temperature rose from 104° to 104.9° F., the conjunctivæ were inflamed, and the leucocyte count was falling. On the 9th day the animal appeared quiet; a few characteristic spots appeared on the mucous membrane of the lips. On the 10th day the temperature had risen to 106.3° F.; there was a well developed, hyperemic, granular, punctate rash on the labial mucous membrane and a few, red maculopapules had appeared on the face. The animal was killed for further transfer of the infection.

*Autopsy.*—No gross abnormalities. Histological sections of the skin, tongue, and labial mucous membrane show the typical lesions of measles. Aerobic and anaerobic cultures of the heart's blood, spleen, and lung showed no growth.

Monkey 14 (Text-fig. 5, c); *Macacus rhesus*. May 20, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, liver, and lung) from Monkey 11 as in the above experiment. The animal remained well for 7 days. On the 8th day it was quiet, showed conjunctivitis and photophobia, and the mucous membranes were congested. No spots were noted. On the 10th day it was drowsy and there was a well developed characteristic exanthem. On the 11th day diarrhea developed; a few small, red maculopapules appeared on the face and thighs. On the 12th day an abundant characteristic exanthem had appeared on the face, neck, chest, shoulders, and thighs. By the 15th day the animal again appeared well; the conjunctivitis and exanthem had cleared up and the exanthem was rapidly fading. Moderate pigmentation persisted for 3 more days and then disappeared. There were a moderate febrile reaction and a well defined leucopenia coincident with the foregoing symptoms. Sections of skin excised from the neck and thigh on the 2nd day of the exanthem show the characteristic histology of measles.

*Second Passage.*—Monkey 16 (Text-fig. 7, a); *Macacus rhesus*. May 29, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, spleen, and lung) from Monkey 13 prepared in the same manner as in the preceding experiment. Culture of the supernatant fluid showed a few colonies of *Staphylococcus albus*. After the customary incubation period of 7 days the animal developed the characteristic symptoms of measles, the course of which is indicated on the temper-



TEXT-FIG. 7, *a* to *d*. Transmission of measles virus, strain from Case 5, from monkey to monkey by means of tissue emulsions. (*a*) Monkey 16, second passage. (*b*) Monkey 19, third passage. (*c*) Monkey 22, fourth passage. (*d*) Monkey 28, fifth passage.

ature chart. The enanthem was very abundant, covering the mucous membranes of the lips and cheeks. The exanthem had appeared on the forehead, nose, chin, and left thigh on the 11th day when the animal was killed for further passage.

*Autopsy.*—Grossly negative except for a few, small, irregular patches of pneumonia about the hilum of the lungs. Culture showed this to be due to a Gram-positive streptococcus of the *viridans* group. Histological sections of the skin and labial mucous membrane show the characteristic lesions of measles.

Monkey 17; *Macacus rhesus*. May 29, 1920. 20 cc. of the tissue emulsion from Monkey 13 were centrifuged at low speed for 10 minutes, the supernatant fluid was diluted with 40 cc. of salt solution and then passed through a Berkefeld N filter (15 minutes, 600 mm. vacuum). Filtration was slow and after about 20 cc. had passed through it was stopped. 10 cc. of the filtrate were inoculated intratracheally. The monkey remained free from definite symptoms and was discarded as negative after 21 days observation.

*Third Passage.*—Monkey 18; *Macacus rhesus*. June 8, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion (skin, mucous membrane of mouth, spleen, and lung) from Monkey 16. Cultures of the emulsion showed a few colonies of *Streptococcus viridans* and *Staphylococcus albus*. This animal promptly developed a severe pneumonia with *Streptococcus viridans* septicemia followed by multiple arthritis and died on the 21st day without exhibiting any definite evidence of measles.

Monkey 19 (Text-fig. 7, b); *Macacus rhesus*. June 8, 1920. Intratracheal injection of 10 cc. of unfiltered tissue emulsion from Monkey 16 as described above. This animal also developed pneumonia but the disease was mild in character. Blood culture on the 5th day showed no growth and the monkey appeared to have recovered. On the 7th day a few, discrete, hyperemic spots appeared on the mucous membrane of the lips. On the 8th day the animal was quiet; the conjunctivæ were injected; fresh spots had appeared on the mucous membranes of the cheeks. On the 9th day there was an abundant, bright red, granular rash on the mucous membranes of the lips, gums, and cheeks. On the 10th day a few, red maculopapules appeared about the lips, on the chin, and behind the ears. The animal was killed for further passage.

*Autopsy.*—Grossly negative except for a small patch of pneumonic consolidation in the right lower lobe. Cultures of this area showed *Streptococcus viridans*. Histological sections of the tongue and labial mucous membrane show the typical lesions of measles.

*Fourth Passage.*—Monkey 22 (Text-fig. 7, c); *Macacus rhesus*. June 17, 1920. Intratracheal injection of 6 cc. of unfiltered tissue emulsion (skin and mucous membranes of lips and cheeks) from Monkey 19. Cultures of the tissue emulsion showed a few colonies of *Staphylococcus albus*, *Streptococcus viridans*, and diphtheroid bacilli. The animal remained well for 7 days. On the 8th day it was quiet; several small, discrete, hyperemic macules with whitish centers appeared on the mucous membrane of the lips. On the 11th day a red, granular rash ap-

peared on the gums. On the 12th day there were moderate conjunctivitis and an abundant enanthem on the mucous membranes of the lips, gums, and cheeks which appeared deeply congested, granular, and covered with minute whitish points. A few, small, red, maculopapules developed about the lips. On the 13th day the exanthem had spread to the arms, abdomen, thighs, and lower legs. The animal was killed for further passage. Blood cultures made daily from the 2nd to 10th days showed no growth.

*Autopsy.*—Grossly negative. Sections of skin, mucous membrane of the lips, and tongue show the typical histology of measles. Cultures of the heart's blood showed no growth.

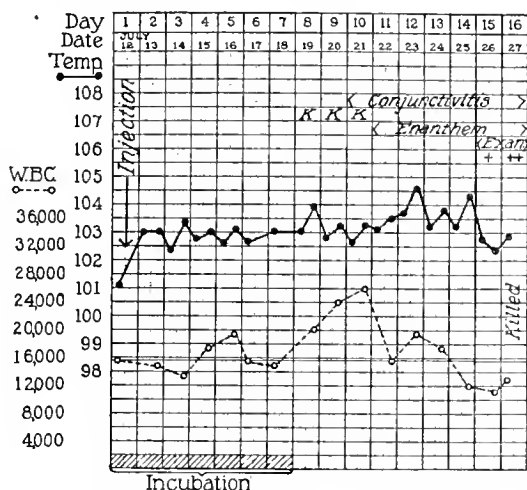
Monkey 23; *Macacus rhesus*. June 17, 1920. Mucous membranes of nose and throat inoculated with 10 cc. of unfiltered tissue emulsion (skin and buccal mucous membrane) from Monkey 19. The animal remained well for 6 days. From the 7th to 14th days it appeared quiet and drowsy. It showed a mild conjunctivitis from the 8th to 14th days with photophobia on the 10th, 11th, and 12th days. A few, discrete, hyperemic macules were present on the mucous membrane of the lips from the 7th to 10th days. No diffuse enanthem and no exanthem appeared at any time. The leucocyte count was low from the 6th to 16th days, varying between 9,460 and 15,740 cells per c.mm., while previous and subsequent counts ranged from 17,460 to 22,800 per c.mm. Although it seemed probable that this animal suffered from a mild infection, the clinical evidence was insufficient to warrant a definite conclusion. Histological sections of the labial mucous membrane, however, show the typical lesion of measles.

*Fifth Passage.*—Monkey 28 (Text-fig. 7, d); *Macacus rhesus*. June 29, 1920. Intratracheal injection of 6 cc. of unfiltered tissue emulsion (skin and mucous membranes of lips and cheeks) from Monkey 22. Cultures of the emulsion showed a few colonies of *Staphylococcus albus*, diphtheroid bacilli, and a Gram-negative diplococcus. The animal remained well for 9 days. On the 10th day it was listless; the conjunctivæ were injected; a small hyperemic spot appeared on the mucous membrane of the upper lip. On the 11th day several discrete spots of similar character appeared on the mucous membrane of the lower lip. On the 12th day there were a well developed characteristic enanthem and a faint eruption of discrete, red maculopapules over the chin, neck, and inner surfaces of the thighs. On the 13th day there was an abundant, well developed, typical exanthem over the face, neck, shoulders, chest, thighs, and back of lower legs. On the 14th day the condition was the same and the animal was killed for further passage.

*Autopsy.*—Grossly negative. Cultures of heart's blood showed no growth. Sections of skin, tongue, and labial mucous membrane show the typical histological picture of measles.

*Sixth Passage.*—Monkey 29 (Text-fig. 8); *Macacus rhesus*. July 12, 1920. Intratracheal injection of 8 cc. of unfiltered tissue emulsion (skin, mucous membranes of lips and cheeks, and piece of tongue) from Monkey 28. Cultures of emulsion showed a few colonies of *Staphylococcus pyogenes aureus*, a non-hemo-

lytic streptococcus, and a Gram-negative bacillus. The animal remained well for 7 days. On the 8th day the temperature rose from 102.9° to 103.8° F.; four discrete hyperemic spots were present on the mucous membrane of the upper lip. On the 10th day diarrhea developed; the conjunctivæ were moderately congested. On the 11th day the conjunctivitis was more marked; a red, granular, punctate rash was present on the mucous membranes. On the 14th day the enanthem was very marked, involving the mucous membranes of lips and cheeks. There were marked conjunctivitis and photophobia. On the 15th day a few, discrete, red maculopapules appeared about the lips. On the 16th day the exanthem was more marked, but had not spread. The enanthem was fading. The temperature had returned to normal. Killed for further passage.



TEXT-FIG. 8. Observations on Monkey 29, sixth passage of measles virus, strain from Case 5.

*Autopsy.*—Grossly negative. Cultures of heart's blood showed no growth. Histological sections of skin and labial mucous membrane show the typical lesions of measles.

*Seventh Passage.*—Monkey 30; *Macacus rhesus*. July 27, 1920. Intratracheal injection of 8 cc. of unfiltered tissue emulsion (skin and mucous membrane of lips) from Monkey 29. This monkey showed little evidence of infection. It was killed on the 14th day, however, in an unsuccessful attempt at further passage. It seems probable that Monkey 29 was killed too late for successful transmission.

The foregoing experiments have shown that the characteristic group of symptoms which occurs in monkeys following inoculation with nasopharyngeal washings from patients with measles is readily

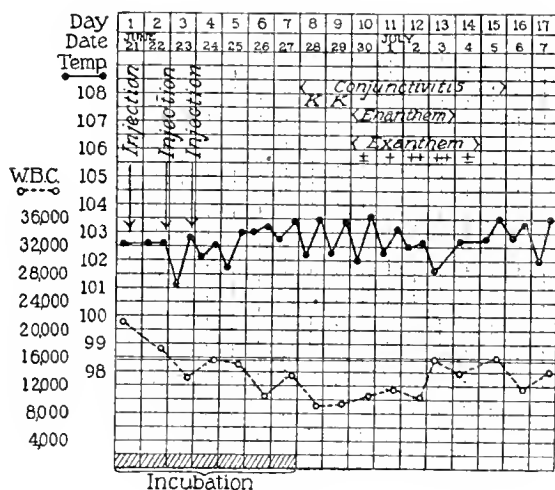
induced in monkeys by intratracheal injection of salt solution emulsions of the skin and buccal mucous membranes of monkeys killed early in the course of the reaction. The transmission of this group of symptoms through six animal passages makes it highly probable that the reaction is due to the living virus of measles. In the use of unfiltered emulsions of the skin and buccal mucous membranes, as in the use of unfiltered nasopharyngeal washings of patients, other organisms than the virus of measles were unavoidably introduced into the trachea. Although there is no evidence that these bacteria were in any way associated with the characteristic measles reaction, it is obviously desirable to use a method of transmission that will exclude the presence of extraneous organisms as a possible source of error in the interpretation of the results obtained. This was attempted in the case of Monkey 17 by filtration of the tissue emulsion, but without success, since under the conditions of the experiment the virus apparently either failed to pass the filter or was so diluted that it failed to give rise to infection. The desired result has been accomplished, however, by the use of blood.

*Transmission of Measles from Monkey to Monkey by Intravenous Injection of Citrated Whole Blood.*

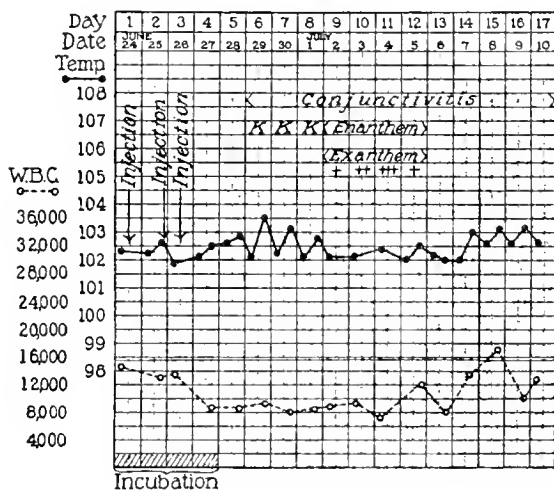
The clinical course and pathological lesions of measles clearly suggest that the virus is present in the blood stream for at least an appreciable length of time. The experiments of Hektoen, Anderson and Goldberger, and Nicolle and Conseil, if the conclusions of the authors are accepted, support this view. To obtain further knowledge on this subject as well as to eliminate the undesirable features of the method of transmission employed in the passage experiments described above, the following experiment was carried out.

*Experiment 6.*—Monkey 22 (Text-fig. 7, c), which had been inoculated intratracheally on June 17, 1920, with 6 cc. of unfiltered tissue emulsion from Monkey 19, was bled daily from the 2nd to 10th days after inoculation and again on the 13th day. The blood, which was received into 2 to 3 cc. of sterile 1 per cent sodium citrate solution, after being cultured, was immediately injected intravenously into other monkeys as follows (Text-fig. 6): The 2nd, 3rd, and 4th day bleedings, in amounts of 10, 7.5, and 12 cc. respectively, were injected into Monkey 24. This





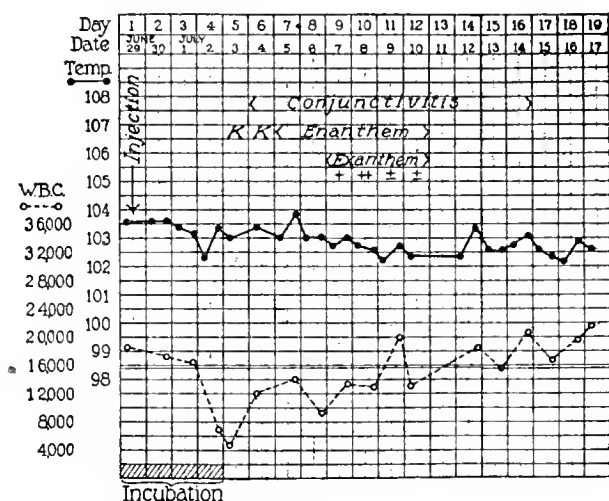
a



b

TEXT-FIG. 9, *a* and *b*. Transmission of measles virus, strain from Case 5, from Monkey 22 by means of whole citrated blood. (*a*) Monkey 25, injected with 5th, 6th, and 7th day bleedings from Monkey 22. (*b*) Monkey 26, injected with 8th, 9th, and 10th day bleedings from Monkey 22.

animal remained free from any symptoms of measles and was discarded as negative after 25 days observation. The 5th, 6th, and 7th day bleedings, in amounts of 9, 8, and 9 cc. respectively, were injected into Monkey 25. This monkey remained well until the 8th day after the first inoculation of blood when it developed the characteristic measles reaction, the course of which is shown in Text-fig. 9, *a*. The 8th, 9th, and 10th day bleedings, in amounts of 12.5, 6, and 13 cc. respectively, were injected into Monkey 26, which fell ill after an incubation period of 4 days with the typical measles symptoms (Text-fig. 9, *b*). The 13th day bleeding, 20 cc., was injected into Monkey 27. This animal likewise showed a typical



TEXT-FIG. 10. Observations on Monkey 27, injected with the 13th day bleeding from Monkey 22. Measles virus, strain from Case 5.

reaction (Text-fig. 10), after an incubation period of 4 days. Cultures of all bleedings from Monkey 22 were made in agar plates. None showed growth. Sections of skin excised from Monkeys 25, 26, and 27, during the period of the exanthem, all show the characteristic histological picture of measles.

The result of the foregoing experiment is important in that it satisfactorily eliminates the objection that can be raised to the preceding passage experiments on the ground that other organisms beside the virus of measles were present in the tissue emulsions employed. It furthermore shows that in monkeys inoculated intratracheally with measles material the virus subsequently enters the blood stream and can be transmitted readily from monkey to monkey

by intravenous injection of whole citrated blood. This observation is of considerable value because it provides a simple method of transmitting the virus from animal to animal in a pure state and affords an opportunity for extensive culture study of a material known to contain the virus of measles and presumably free from other organisms. There are other suggestive points in the experiment which lend support to previous conceptions of the course of measles in man. The failure of Monkey 24 to react suggests that an appreciable period intervenes between the time of infection of the mucous membranes of the respiratory tract and the entrance of the virus into the blood stream. The positive transmission to Monkey 25 shows that the virus had entered the blood of Monkey 22 by at least the 7th day after inoculation, a time which corresponded with the end of the incubation period of the disease and preceded the onset of symptoms by 24 hours. Whether the virus was present in the blood of Monkey 22 on the 5th and 6th days it is impossible to say. It is of interest in this connection that the incubation period in Monkey 25 was 7 days, while in Monkeys 26 and 27 it was but 4 days. This suggests the possibility that the virus was not present in the 5th and 6th day bleedings from Monkey 22. The difference in incubation period, however, may be explained on the assumption that the virus was much more abundant in the blood of Monkey 22 from the 8th to 13th days, which coincided with the active stage of the disease, than from the 5th to 7th days, which coincided with the latter half of the incubation period. The positive results in Monkeys 26 and 27 show that the virus was present in the blood from the onset of the disease until at least the 2nd day of the exanthem. How much longer it may persist in the blood has not as yet been determined. The shortening of the incubation period in Monkeys 26 and 27 from the customary 7 days to 4 days was presumably dependent upon the introduction of the virus directly into the blood stream, since by this method of inoculation the period of several days which probably elapses between the inoculation of the virus on the mucous membranes and its invasion of the blood is eliminated. Although our observations on the infectivity of the blood have so far been made only on the experimental disease in monkeys, it seems probable that similar conditions will be found to obtain in measles in man.

## SUMMARY.

By the intratracheal injection into monkeys of unfiltered nasopharyngeal washings from cases of measles in the preeruptive and early eruptive stages of the disease a relatively constant group of symptoms was induced which closely resemble those of measles in man. Of seven monkeys inoculated intratracheally with unfiltered nasopharyngeal washings from seven cases of measles, five developed the symptoms. The same group of symptoms was induced in one monkey by inoculation of the mucous membrane of the nose and mouth with unfiltered nasopharyngeal washings from a case of measles. In these experiments a variety of organisms, largely saprophytic inhabitants of the nasopharynx and mouth, were present in the material inoculated. There is sufficient evidence, however, that these organisms were in no way responsible for the reaction, since the same group of symptoms was induced in two monkeys by the intratracheal injection of nasopharyngeal washings from three cases of measles after the washings had been freed from ordinary organisms of the mouth flora by filtration through Berkefeld N filters.

The characteristic group of symptoms which follows the inoculation of monkeys with the nasopharyngeal washings from patients with measles has been successfully carried through six passages by intratracheal injection of saline emulsions of the skin and buccal mucous membranes of monkeys killed from 2 to 6 days after the onset of the reaction. From the fourth passage monkey the reaction was also successfully induced in three monkeys by means of citrated whole blood injected intravenously. This experiment showed the blood to be capable of inciting the reaction from at least the 7th to 13th days after intratracheal inoculation of the donor monkey, but incapable of inducing it from the 2nd to 4th days. Cultures of the blood showed no growth.

The group of symptoms induced has been constant and definite in character. After an incubation period of 6 to 10 days the animal becomes listless and drowsy, the conjunctivæ become injected, and small, discrete, hyperemic macules appear on the labial mucous membrane. These spots increase in number and may eventually coalesce in the course of 2 to 4 days to form a diffuse, red, granular

rash. This rash is usually limited to the labial mucous membrane but may extend to the inside of the cheeks. The individual macules may or may not show the minute bluish white center characteristic of Koplik spots. From one to several days after onset an eruption of small, discrete, red maculopapules appears on the skin, usually coming out first on the face. The rash progressively increases in the number and size of the individual lesions and may in the course of 2 to 3 days extend to the skin of the neck, shoulders, upper arms, chest, abdomen, and thighs. It is constant in character but varies considerably in extent in different animals. By the time the exanthem is fully developed, the rash on the mucous membranes has begun to fade and soon disappears. The exanthem in turn progressively fades, sometimes with a branny desquamation, sometimes without. There may be moderate pigmentation. By the 6th to the 10th day after onset all symptoms have disappeared and the animal again appears well. Coincident with this group of symptoms there is a constant and definite reduction in the total leucocyte count, frequently constituting a true leucopenia. Other symptoms of irregular occurrence are photophobia, diarrhea, and fever. Symptoms of rhinitis and bronchitis have not been noted. Histological sections of the lesions of the skin show an exudative and proliferative lesion about the capillaries of the corium in which endothelial leucocytes are the predominating cells. Mitotic cells are not infrequently present in these areas. The endothelial leucocytes may be seen migrating toward and occasionally invading the epithelial layers of the hair follicles, sebaceous glands, and epidermis. In places the epithelial cells appear edematous and vacuolated, and there is evidence of minute vesicle formation. The lesions of the labial mucous membrane are similar in character. Minute vesicle formation in the epithelium is more frequent and the vesicles occasionally assume a more pustular appearance. Similar lesions are found in histological sections of the tongue. Cultures of the blood made both during the incubation period and during the course of the reaction in a variety of media, aerobic and anaerobic, have consistently shown no growth.

The close similarity of the symptoms and pathological lesions of the reaction to the symptoms and pathological lesions of measles, the successful transmission of the reaction from monkey to monkey,

and the elimination of ordinary bacteria as a possible source of error in the interpretation of the results, warrant the belief that the reaction is caused by the inciting organism of measles.

#### CONCLUSION.

Monkeys (*Macacus rhesus*) are susceptible to inoculation with the virus of measles.

#### EXPLANATION OF PLATE 36

FIG. 1. Exanthem in Monkey 10, inoculated with the nasopharyngeal washings from a patient with measles. The drawing illustrates the character and distribution of the lesions on the 3rd day after the first appearance of the exanthem.



FIG. 1.

(Blake and Trask—Studies on measles, 1.)





## STUDIES ON MEASLES.

### II. SYMPTOMATOLOGY AND PATHOLOGY IN MONKEYS EXPERIMENTALLY INFECTED.

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PLATES 37 TO 41.

(Received for publication, November 15, 1920.)

It has already been shown<sup>1</sup> that a group of symptoms closely resembling those of measles is readily induced in monkeys by inoculation with the nasopharyngeal secretions of patients in the pre-eruptive or early eruptive stages of measles, and that the virus inducing this reaction is readily transmitted from monkey to monkey by inoculation with blood or tissue emulsions from monkeys experimentally infected. In this paper the symptoms and course of the reaction and the histology of the lesions of the skin, labial mucous membrane, and tongue will be described.

#### *Symptomatology.*

*Incubation Period.*—In monkeys inoculated on the mucous membranes of the respiratory tract the incubation period is remarkably constant, being 7 days in the majority of animals. It may vary, however, from 6 to 10 days. Thus in sixteen monkeys inoculated on the respiratory mucous membrane, the incubation period was 6 days in four, 7 days in nine, 8 days in one, 9 days in one, and 10 days in one. During this period the animal appears well and exhibits no recognizable symptoms. The incubation period after intravenous inoculation is apparently somewhat shorter (4 days), but enough animals have not been inoculated by this method to warrant a definite statement.

<sup>1</sup> Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 385.

*Onset and Course.*—The onset is indicated by listlessness, loss of appetite, drowsiness, and diminution in the total leucocyte count. There may be a sharp rise in temperature, but this is not constant. The conjunctivæ become injected, and small, discrete, hyperemic macules appear on the labial mucous membrane. During the following 2 or 3 days these increase in number and may eventually coalesce to form a diffuse, hyperemic enanthem on the mucous membrane of the lips and cheeks. From 1 to 5 days after onset a red, maculopapular rash appears on the skin, usually coming out first on the face. The rash increases and may eventually spread to the skin of the neck, arms, chest, abdomen, and thighs, reaching its height in 2 or 3 days. By this time the enanthem is fading. The exanthem in turn progressively fades, and by 6 to 10 days after onset all symptoms have disappeared.

*Conjunctivitis.*—An acute catarrhal conjunctivitis is exhibited by nearly all animals during the period of the reaction. This conspicuously involves the inner canthus and tarsal conjunctiva, the bulbar conjunctiva remaining little if at all affected. It varies from a moderate congestion of the vessels to a diffuse hyperemia. In the more severe cases there are increased mucoid secretion, increased lachrimation, and definite photophobia. Moderate edema of the lids may be present. Purulent inflammation does not occur.

*Enanthem.*—Coincident with or shortly following the onset of the reaction a definite and characteristic enanthem appears. This begins with the development of one or more discrete, red spots on the labial mucous membrane. These spots usually occur only on the labial mucosa, but may occasionally be present on the inside of the cheeks and on the gums. They do not occur on other parts of the buccal mucosa. They present the appearance of discrete, bright red, slightly elevated, lusterless macules, 1 to 2 mm. in diameter. Occasionally they show a minute, bluish white center. They occur either singly or in small groups of two or three. Their number progressively increases during the first 2 or 3 days of the reaction. By the end of this time there may be ten to fifteen in all. From this point the enanthem pursues one of two courses. In the milder infections the spots now begin to fade and in 1 to 2 days have disappeared. In more severe infections, however, the spots coalesce to form a more

diffuse, red, slightly granular rash covering large areas of the labial mucous membrane with intervening areas of pale pink mucosa which appear to be normal. In still severer infections the entire labial mucous membrane and the inside of the cheeks finally become covered with a diffuse, bright red, granular rash studded with numerous minute, white, slightly depressed specks. The enanthem now begins to fade and rapidly disappears in the course of 2 to 3 days.

*Exanthem.*—From 1 to 5 days, usually on the 3rd or 4th day, after onset a characteristic exanthem appears. It generally comes out first on the face, especially about the eyes, the corners of the mouth, and on the cheeks, but may also appear approximately at the same time on other parts of the body, particularly on the inner surface of the thighs. It begins with a few, discrete, red maculopapules occurring either singly or in small groups. The individual maculopapules are 2 to 3 mm. in diameter, fade out gradually at their margins into the surrounding normal skin, and are frequently located about a hair follicle. They disappear on pressure and are never hemorrhagic. There is no diffuse erythema of the intervening skin, which appears normal. The further progress of the rash shows the characteristic evolution of the measles exanthem, though the rash itself rarely becomes as widespread or as thick as it commonly does in man. Generally in the course of 2 or 3 days the rash comes out progressively on the skin of the neck, chest, lower abdomen, and the inner surfaces of the upper arms and thighs. While it is usually limited to these less hairy areas of the skin, it may occasionally be sparsely present on the scalp, shoulders, back, forearms, and posterior surface of the legs. During this period the individual maculopapules tend to increase in size, but they never become sufficiently large or thickly aggregated to produce a confluent rash. Having reached its height the rash begins to fade and disappears in the course of 2 to 5 days. This sometimes takes place without noticeable desquamation, but there frequently is a fine, branny desquamation of the skin over the central portion of the maculopapules. Occasionally a slight, yellowish brown pigmentation may persist for 2 or 3 days after the complete fading out of the hyperemia. While the foregoing description presents a composite picture of the exanthem, the extent of the rash and the number of maculopapules vary considerably in individual animals.

Thus it may vary from a comparatively sparse exanthem limited to the face or face and inner surfaces of the thighs, to a widespread, moderately thick exanthem involving the skin of the face, neck, trunk, and extremities. The character of the individual maculopapules, however, remains constant and typical.

*Fever.*—There is the widest possible variation in febrile reaction. The temperature may rise abruptly at onset, even to as high as 105–106°F. On the other hand, the disease may begin without fever and remain so throughout. When fever occurs it may persist only during the prodromal stage before the appearance of the exanthem or may continue throughout the course of the disease. It may be distinctly remittent in type or fairly well sustained. In an occasional animal the preliminary rise at onset is followed by a return to normal and a distinct secondary rise. In these cases the temperature curve closely resembles that of measles in man.

*Leucopenia.*—A definite diminution in the total leucocyte count consistently occurs coincident with the reaction. This usually begins 1 or 2 days before the onset of symptoms and persists for several days until the height of the exanthem is reached. At this time the leucocytes begin to increase and gradually return in the course of 3 days to a week to their former level. The degree of leucopenia varies somewhat in different animals, but it is nearly always well defined and may be very marked, many animals showing counts as low as 4,000 to 9,000 cells per c.mm. over a period of several consecutive days. No significant change in the leucocyte count which may be attributed to the virus of measles appears to occur during the earlier part of the incubation period. Animals inoculated with unfiltered nasopharyngeal washings usually show a leucocytosis for 24 to 48 hours after inoculation, but this is undoubtedly due to the effect of other organisms, since it does not occur in animals inoculated with filtered washings in which other organisms than the virus of measles are presumably not present. It has not as yet been determined whether the diminution in leucocytes is due to a greater reduction in one type of leucocyte as compared with other types.

*Other Symptoms.*—The general symptoms of malaise, such as loss of appetite and activity, are usually moderate in degree, but may occasionally be quite marked. They begin with the onset of the

reaction and persist until the exanthem begins to fade. A very definite drowsiness is often present during the first 2 or 3 days of the disease. Another symptom of not infrequent occurrence is a moderate diarrhea during the earlier part of the reaction. The entire absence of symptoms of rhinitis and bronchitis should be noted.

*Microscopic Pathology of Skin, Labial Mucous Membrane, and Tongue.*

The tissues available for study consisted of small pieces of skin excised,<sup>2</sup> during life from twelve monkeys during the period of the exanthem and pieces of skin, labial mucous membrane, and tongue obtained at autopsy from seven monkeys killed for passage of the virus. The tissues were fixed in Zenker's fluid and stained with alum-hematoxylin and eosin. A composite picture of the lesions will be given.

*Skin.*—Lesions are present both in the corium and in the epidermis. The reaction in the corium (Figs. 1 to 6) is definite and typical. It consists of swelling and proliferation of the endothelial cells lining the capillaries and smaller veins, accumulation of endothelial leucocytes<sup>3</sup> about the capillaries (Figs. 2, 3, and 5), and active multiplication of these emigrated leucocytes. In addition there is a moderate exudation of serum into the pericapillary tissue (Figs. 1 and 3). In the earlier lesions a very few eosinophils, polymorphonuclear leucocytes, and lymphocytes may also be present in the pericapillary exudate. Diapedesis of red blood corpuscles has not been seen. The endothelial cells of the capillary walls appear swollen; their cytoplasm is finely granular. Occasionally one is seen in mitosis (Fig. 1). The emigrated endothelial leucocytes are young and active. Their nuclei are frequently lobulated. In early cases these leucocytes are in active mitosis (Figs. 1, 2, and 4), as many as three to five mitotic cells sometimes being present about a capillary. Occasionally they

<sup>2</sup> This was always done under anesthesia.

<sup>3</sup> The term endothelial leucocyte is used for the sake of convenience in comparing the lesions in monkeys with the lesions of human measles as recently described by Mallory and Medlar.<sup>5</sup> The authors, however, do not wish to commit themselves to the opinion that the wandering mononuclear phagocytes are derived solely from endothelial cells and not in part from other fixed tissue cells of mesenchymal origin.

show phagocytosis of polymorphonuclear leucocytes and lymphocytes. In later lesions the exudation of serum is less marked, mitotic cells are no longer present, and polymorphonuclear leucocytes are not seen.

These lesions are chiefly in the upper part of the corium and not infrequently lie close to the hair sheaths and sebaceous glands. Although the exudate is primarily pericapillary in location, it also extends into and involves the epithelium of the epidermis, hair sheaths, and sebaceous glands when the capillary lesions lie closely adjacent to these structures.

The lesions in the epidermis (Figs. 6 to 9) are less numerous and conspicuous than those in the corium. In early cases there are minute foci of serous exudate in the epidermis leading to swelling and vacuolation of the epithelial cells of the Malpighian layer (Figs. 6 and 7). The nuclei of these cells are distorted and may appear crescent-shaped and pycnotic. The serum sometimes accumulates under the cornified layer, producing minute vesicles (Figs. 8 and 9). There is slight infiltration of these foci with endothelial leucocytes. Occasionally a more diffuse infiltration of the epidermis with endothelial leucocytes takes place, but this is never very conspicuous. These early exudative lesions quickly give way to retrograde changes. The epithelial cells either singly or in minute clumps show necrosis, the serous exudate disappears, and minute, thickened plaques are present in or beneath the cornified layer.

Lesions similar to those in the epidermis are seen in the hair sheaths and in the sebaceous glands when they lie close to a capillary lesion. Infiltration with endothelial leucocytes is apt to be more conspicuous than in the epidermal lesions and may result in the appearance of minute pustules in these structures.

*Labial Mucous Membrane.*—The lesions in the labial mucous membrane (Figs. 10 to 13) are essentially the same as those occurring in the skin. There are swelling and proliferation of the capillary endothelium (Fig. 10), exudation of serum, and migration of endothelial leucocytes into the pericapillary tissue (Fig. 10), and multiplication of the emigrated leucocytes. A very few polymorphonuclear leucocytes, eosinophils, and lymphocytes are also present in early lesions. Invasion of the epithelium is similar to that of the epidermis, but

usually more marked. It consists of small foci of serum and endothelial leucocytes beneath the stratified epithelium with vacuolation and necrosis of the epithelial cells (Figs. 11 and 12). Diffuse infiltration of the epithelium by endothelial leucocytes is sometimes quite marked. With the progress of the lesion the stratified epithelium covering these minute pustules macerates and sloughs off leaving minute shallow ulcerations on the surface (Fig. 13). At this stage the minute lesions in the epithelium not infrequently show a considerable number of polymorphonuclear leucocytes, presumably due to secondary infection.

*Tongue.*—The lesions in the mucous membrane of the tongue (Fig. 14) are identical with those in the labial mucosa. The capillaries supplying the papillæ show swelling and proliferation of the endothelium, there are exudation of serum and endothelial leucocytes into the stroma, and invasion of the epithelium by serum and endothelial leucocytes with the formation of minute pustules beneath the stratified layers.

#### DISCUSSION.

That the symptoms observed in monkeys experimentally inoculated with material containing the virus of measles closely resemble those of human measles seems evident. The incubation period, the conjunctivitis, the enanthem, the exanthem, and the leucopenia closely parallel the similar characteristic features of measles in man. While it is true that the average incubation period in monkeys is 3 to 4 days shorter than it is in man, this might reasonably be expected in view of the presumably large amount of virus inoculated by intratracheal injection. At least it would seem probable that a much larger amount of virus than that which serves to transmit measles from man to man in the natural spread of the disease, is present in 5 to 10 cc. (the amount used) of nasopharyngeal washings from a patient or of tissue emulsion from an infected monkey. If this is so, and there is no evident reason for believing that it is not, the somewhat shorter incubation period is not surprising. The conjunctivitis in monkeys as in man is catarrhal rather than purulent in character, and like that of the human disease it is not infrequently accompanied by definite photophobia and increased lacrimation.

The early, discrete macules on the buccal mucous membrane correspond in time of appearance and in distribution with Koplik spots and differ from them only in the inconstant presence of the characteristic minute, bluish white center. The further evolution of the rash on the buccal mucous membrane is essentially identical with the evolution of the measles enanthem. The exanthem in time of appearance, in the character of the individual lesions, and in evolution likewise closely parallels the exanthem of measles and differs from it only in that it is usually more sparse and less widespread. In fact, there would appear to be only one conspicuous difference between the experimental disease and human measles; namely, the absence of respiratory symptoms in the experimental infection. While this difference cannot be explained at present, it would not appear to constitute a valid reason for rejecting the belief that the reaction observed in experimentally infected monkeys is caused by the virus of measles.

Further and even more conclusive evidence is found in the character of the lesions in the skin and in the buccal mucous membrane. These lesions have been carefully studied in cases of human measles by Ewing<sup>4</sup> and more recently by Mallory and Medlar.<sup>5</sup>

According to the latter authors the lesions in the skin are "due to a proliferative and exudative reaction in and around a small network of capillaries in the upper part of the corium. The reaction consists of occasional mitoses in the lining endothelial cells; of emigration of endothelial leucocytes, and of an active proliferation of them around the vessels; of a very slight emigration of polymorphonuclear leucocytes and lymphocytes; and of phagocytosis of them by the endothelial leucocytes.

"The exudation of serum and endothelial leucocytes at first is active, and passes to the adjoining epidermis, hair sheaths and sebaceous glands. It often collects in small foci, forming minute vesicles and pustules. The epithelial cells involved in the exudation undergo necrosis. By the time the exanthem is clearly evident, these minute lesions in the epidermis are already beginning to dry up, and later desquamate as scales. In the meantime the endothelial leucocytes in the corium continue to proliferate and accumulate around the blood vessels for two to four days, and then gradually disappear.

"Koplik spots correspond exactly to the minute early lesions of the epidermis.

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<sup>4</sup> Ewing, J., *J. Infect. Dis.*, 1909, vi, 1.

<sup>5</sup> Mallory, F. B., and Medlar, E. M., *J. Med. Research*, 1920, xli, 327.



but instead of drying up they tend to macerate, and may terminate in erosions or, if secondarily infected, in ulcerations."

It is clear from the foregoing description that the lesions of the skin and buccal mucosa in human measles are exactly like those found in the skin and buccal mucous membrane of the experimental infection induced in monkeys.<sup>6</sup>

#### SUMMARY AND CONCLUSIONS.

The symptomatology of the reaction induced in monkeys by inoculation with material containing the virus of measles is described. The symptoms and course of this reaction closely parallel those of human measles.

The microscopic pathology of the lesions of the skin and buccal mucous membrane of monkeys experimentally infected with the virus of measles is also described. These lesions are essentially identical with the corresponding lesions of measles in man.

#### EXPLANATION OF PLATES.

##### PLATE 37.

FIG. 1. Early lesion in the corium showing mitosis of an endothelial cell lining a capillary, and pericapillary exudate of serum and endothelial leucocytes, one of which is in mitosis.  $\times 1,000$ .

FIG. 2. Endothelial leucocytes accumulated about a capillary in the corium; one is shown in mitosis.  $\times 1,000$ .

##### PLATE 38.

FIG. 3. Early lesion about a small vein in the corium showing an exudate of serum and endothelial leucocytes.  $\times 1,000$ .

FIG. 4. Two endothelial leucocytes in mitosis near a capillary in the corium.  $\times 1,000$ .

FIG. 5. Exudate of serum and endothelial leucocytes in the corium.  $\times 500$ .

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<sup>6</sup> We wish to express our gratitude to Dr. Mallory for being so kind as to examine histological sections of the skin and buccal mucosa from the infected monkeys. We are also indebted to him for permission to quote his opinion that the lesions in the monkeys present essentially the same histological picture that is found in the corresponding lesions of measles in man.

## PLATE 39.

FIG. 6. Accumulation of endothelial leucocytes about capillaries and small veins in the corium; focal infiltration of the epidermis with serum and endothelial leucocytes.  $\times 240$ .

FIG. 7. Early lesion in the epidermis showing invasion of the Malpighian layer by endothelial leucocytes and beginning vacuolation.  $\times 1,000$ .

## PLATE 40.

FIG. 8. Early vesicle formation in the epidermis.  $\times 240$ .

FIG. 9. Vesicle in the epidermis with beginning plaque formation in the cornified layer.  $\times 240$ .

FIG. 10. Accumulation of endothelial leucocytes near two capillaries in the labial mucosa; swelling of the capillary endothelium.  $\times 1,000$ .

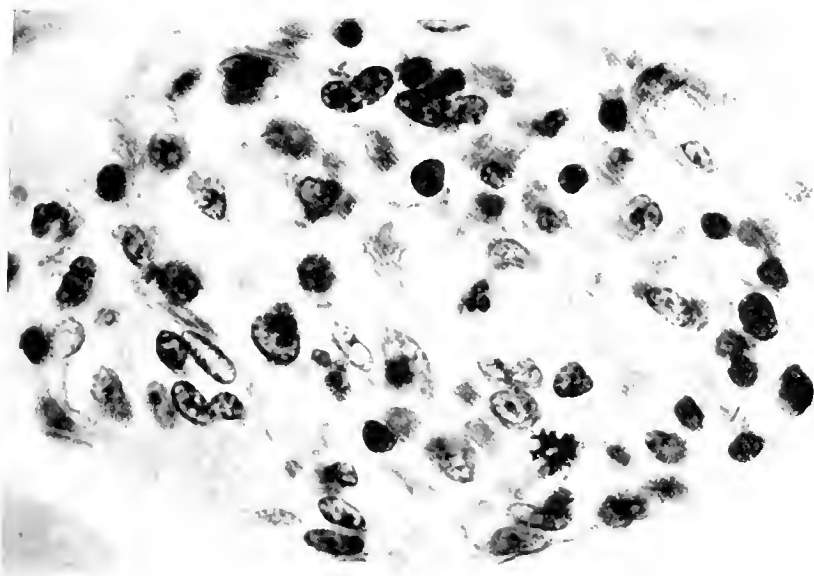
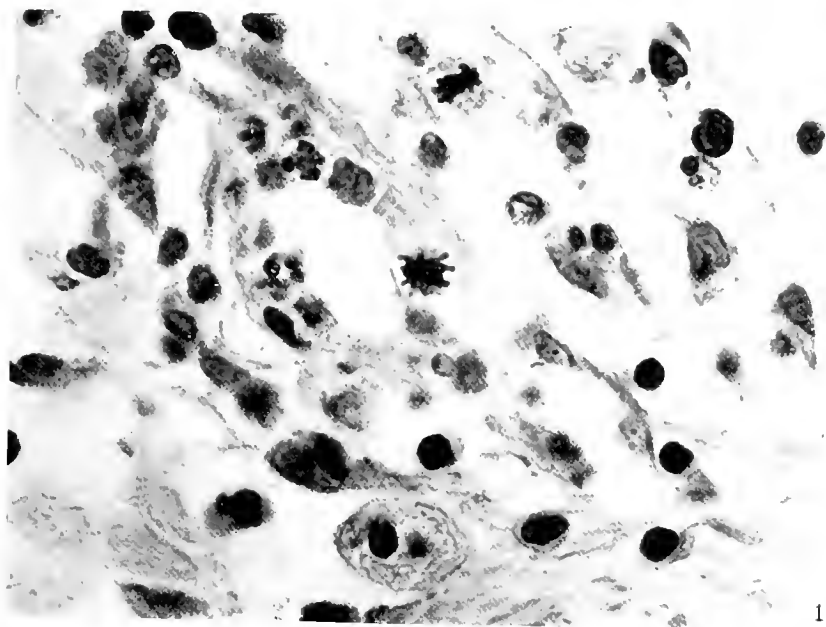
## PLATE 41.

FIG. 11. Koplik spot in the labial mucosa, very early stage; focal infiltration of the epithelium with serum and endothelial leucocytes.  $\times 1,000$ .

FIG. 12. Koplik spot in the labial mucosa, early stage; focal accumulation of serum and endothelial leucocytes in the epithelium (pustule formation); degeneration and necrosis of epithelial cells.  $\times 240$ .

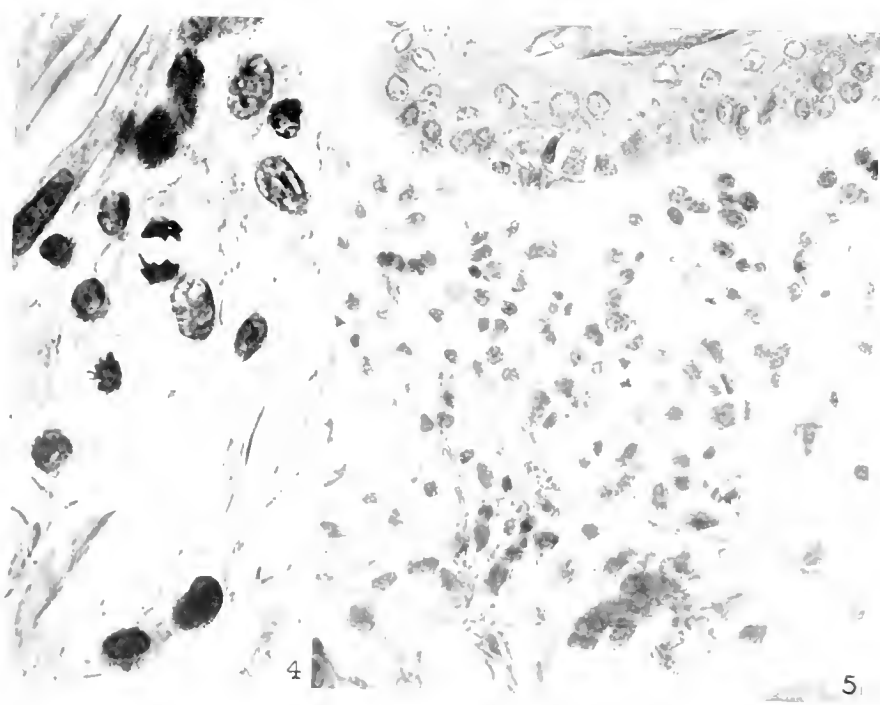
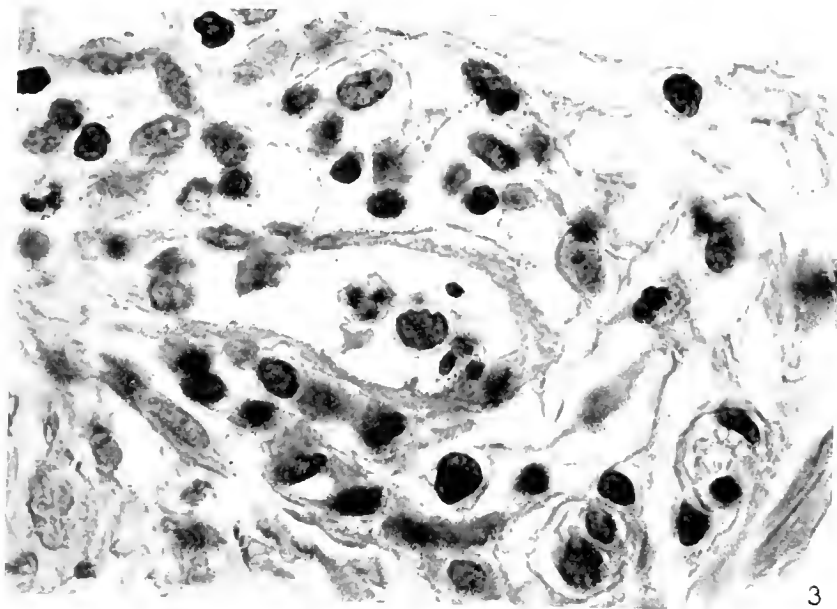
FIG. 13. Koplik spot in the labial mucosa, late stage; necrosis and maceration of the epithelium with erosion of the surface.  $\times 240$ .

FIG. 14. Diffuse infiltration of the epithelium of the tongue with endothelial leucocytes.  $\times 240$ .

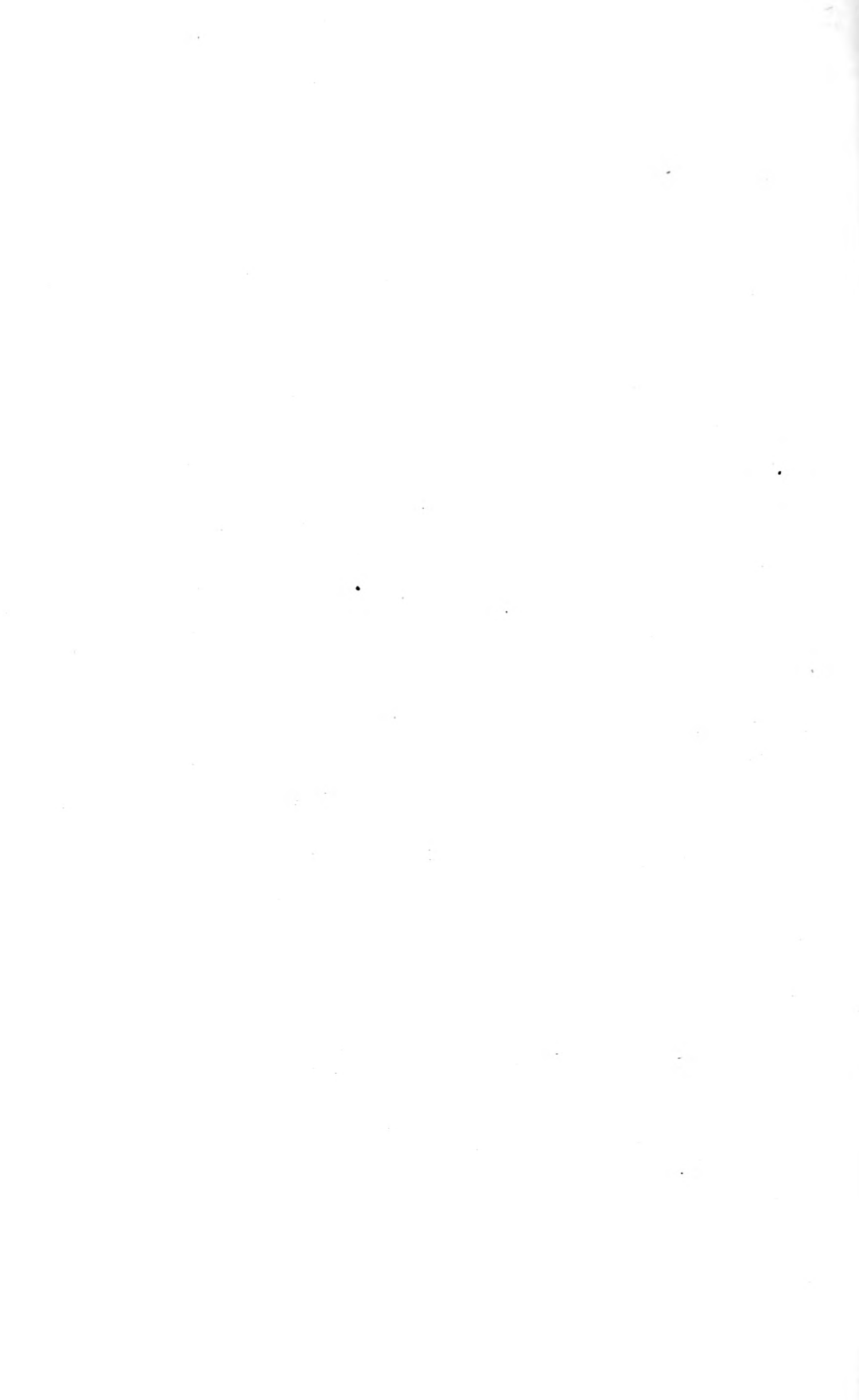


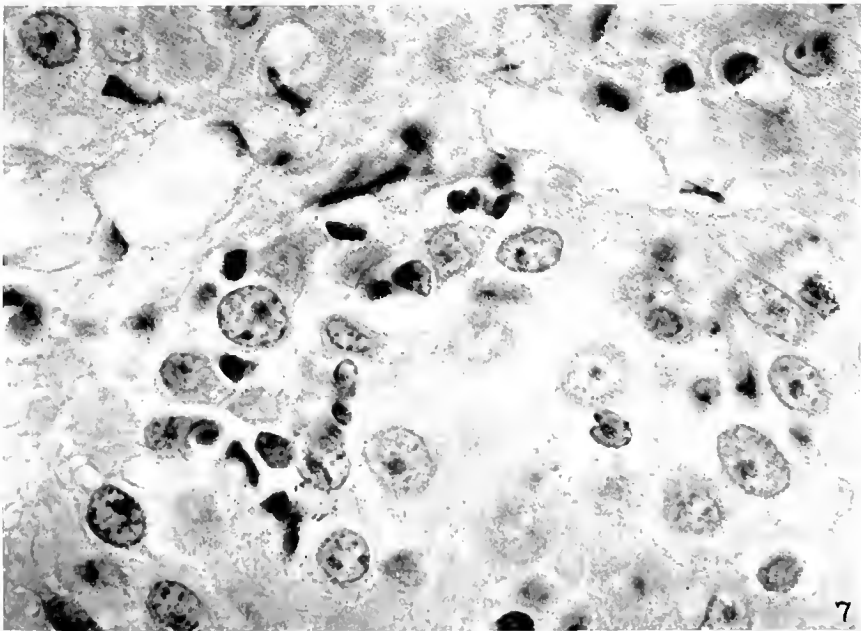
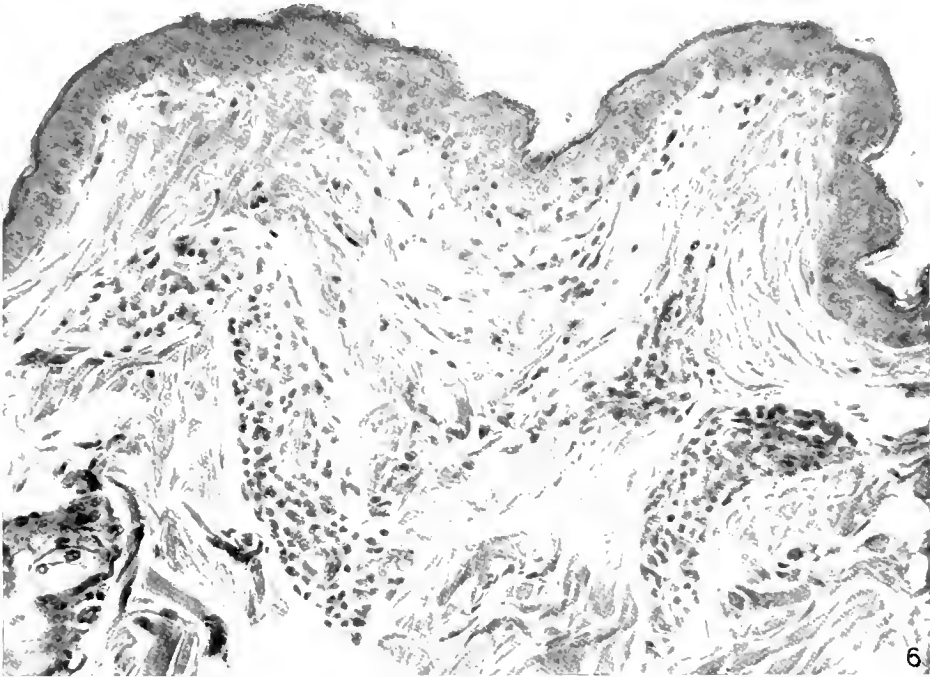
(Blake and Trask: Studies on measles. II.)





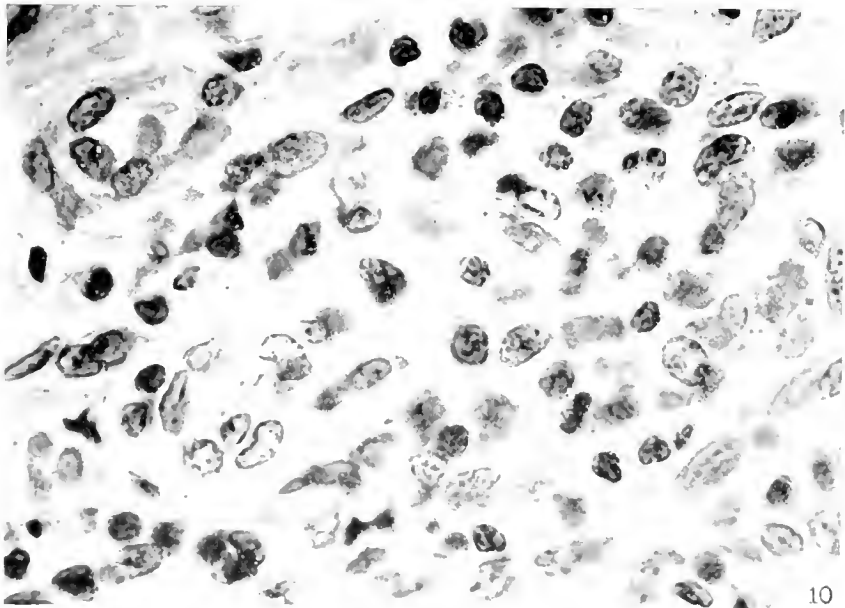
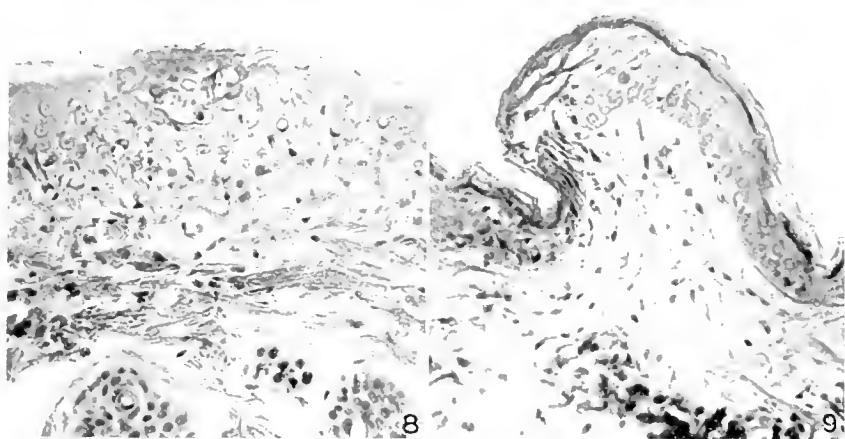
Blake and Trask: Studies on measles. (11)



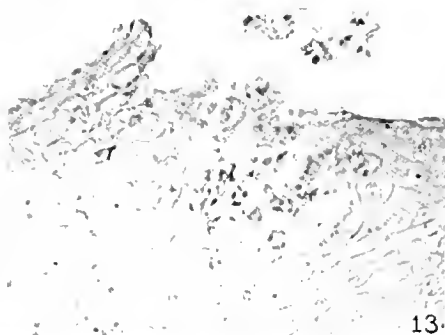
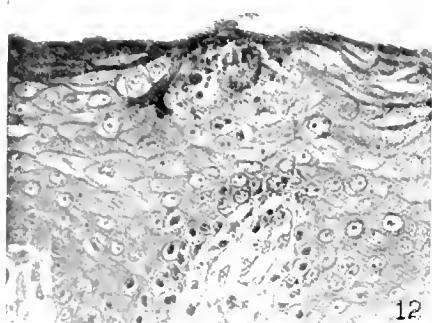
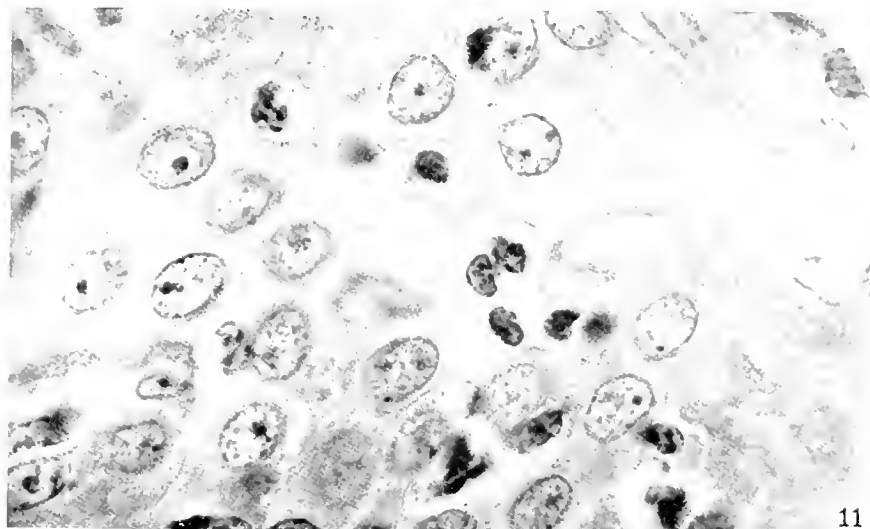














## STUDIES ON LYMPHOID ACTIVITY.

### V. RELATION BETWEEN THE TIME AND EXTENT OF LYMPHOID STIMULATION INDUCED BY PHYSICAL AGENTS AND THE DEGREE OF RESISTANCE TO CANCER IN MICE.

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(Received for publication, December 31, 1920.)

Two methods have been described by which a definite stimulation of the circulating lymphocytes, accompanied by a hyperactivity of the lymphoid centers, may be brought about. It was first noted that small doses of x-rays would induce this condition,<sup>1</sup> but the reaction was of short duration<sup>2</sup> as compared with the stimulation occurring in cancer-immune mice following inoculation of cancer.<sup>3</sup> The stimulation induced by x-rays is preceded by a period during which evidences of the destructive action of this agent on the lymphoid centers are present, and is followed by a period during which the stimulation phase alone is present. By the 4th day the proliferative activity is at its height and then quickly subsides.

The amount of stimulus produced by dry heat<sup>4</sup> is much greater in extent and of longer duration than that seen after exposure to x-rays. Immediately after the heat application there are also marked evidences of cell destruction in the lymphoid centers, but the stimulation following is more prompt and of greater volume as judged by the blood pictures.

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

<sup>2</sup> Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

<sup>3</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.

<sup>4</sup> Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

We have described the close relation existing between lymphoid stimulation and the resistant state to cancer inoculation. If this relation is quantitative in nature, it would be expected that mice whose lymphoid cells are stimulated by x-rays would show a definite degree of resistance but less than that exhibited by animals following heat stimulation. This fact is borne out by our figures on 102 mice heated 1 week before inoculation, which showed an average immunity of 60.3 per cent, controlled by 83 normal mice inoculated with the same tumors showing an average immunity of 16.5 per cent. On the other hand, 144 mice, x-rayed from 3 to 7 days before inoculation, had an immunity of 37.5 per cent; they were controlled by 137 normal mice inoculated with the same tumors, having an average immunity of 10.4 per cent.

If the hypothesis is true that stimulation of the lymphocytes of mice definitely reduces the number of takes of cancer grafts, it would be of importance to know whether there is a difference in resistance percentage when the tumor inoculation is made at varying times after the stimulus is administered. The following experiments were planned to test this point.<sup>5</sup>

#### *Immunity after Exposure to X-Rays.*

Mice of about the same age were divided into three lots. Two of these lots were given a dose of x-rays governed by the following factors: spark-gap  $\frac{3}{8}$  inch between points, milliamperage 25, distance from target to back of animals 8 inches, and time 10 minutes. One lot received this dose 7 days and the other immediately before they were inoculated with a tumor (Bashford 63). At the same time the third group was inoculated with the same material and weekly measurements were made of the resultant tumors.

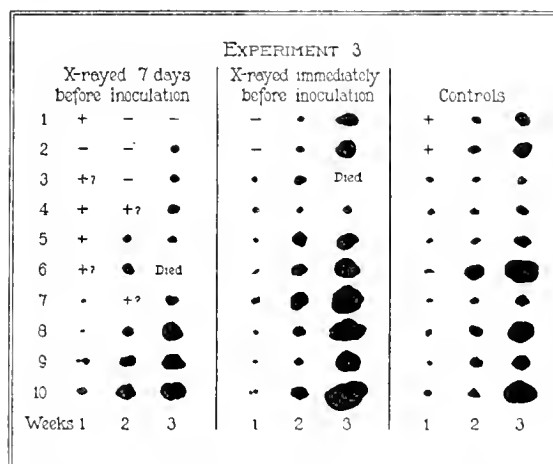
Table I gives the results of six experiments, the percentage of immune mice being estimated for a period 3 weeks after inoculation (Text-figs. 1 and 2).

<sup>5</sup>A preliminary report of this work was made before the National Academy of Science (Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35).

TABLE I.

Experiment No.	Group 1.	Group 2.	Group 3.
1	11.1 per cent ( 9 mice).	0.0 per cent (10 mice).	0.0 per cent (10 mice).
2	30.0 " " (10 " ).	12.5 " " ( 8 " ).	10.0 " " (10 " ).
3	10.0 " " (10 " ).	0.0 " " (10 " ).	0.0 " " (10 " ).
4	50.0 " " (10 " ).	10.0 " " (10 " ).	20.0 " " (10 " ).
5	40.0 " " (10 " ).	11.1 " " ( 9 " ).	11.1 " " ( 9 " ).
6	50.0 " " (10 " ).	0.0 " " (10 " ).	10.0 " " (10 " ).

Group 1 was made of mice given a dose of x-rays 7 days before cancer was inoculated. Group 2 animals were x-rayed immediately before the cancer inoculation. Group 3 comprised the control mice inoculated with the same cancers. 175 mice were used for these tests.



TEXT-FIG. 1. The effect of x-rays on the immunity to transplanted cancer when administered 7 days and immediately before inoculation.

From the foregoing experiments it is seen that the resistance of mice inoculated with cancer immediately after a stimulating dose of x-rays is no higher than that of normal mice, and on the average is somewhat lower. On the other hand, the mice inoculated with cancer a week after a stimulating dose of x-rays show a consistently higher degree of resistance which reveals itself both in the number of takes and in the rate of growth of the tumor.

EXPERIMENT 6									
X-rayed 7 days before inoculation			X-rayed immediately before inoculation			Controls			
1	-	-	-	+	+	-	-	-	
2	-	-	-	+	+	+	+	+	
3	-	-	-	+	+	+	+	+	
4	+	-	-	+	+	+	+	+	
5	+	-	-	+	+	+	+	+	
6	+	+	+	+	+	+	+	+	
7	+	+	+	+	+	+	+	+	
8	+	+	+	+	+	+	+	+	
9	+	+	+	+	+	+	+	+	
10	+	+	+	+	+	+	+	+	
Weeks 1	2	3	1	2	3	1	2	3	

TEXT-FIG. 2. The effect of x-rays on the immunity to transplanted cancer when administered 7 days and immediately before inoculation.

#### *Immunity after Exposure to Dry Heat.*

These experiments with x-rays have been paralleled with similar tests with heat as the agent for stimulating the lymphocytes.

Three groups of mice were used. Group 1 consisted of animals which had been heated 1 week previous to the inoculation with cancer. Group 2 mice were heated immediately before the cancer inoculation, and Group 3 consisted of the normal controls inoculated with cancer. The mice to be heated were placed in an enclosed cage 3 inches above an electric heating lamp. A thermometer was placed half an inch below the bottom of the cage. The temperature was allowed to rise to 55°C. before the animals were put in the cage and then they were left in for 5 minutes, the temperature rarely rising above 63°C. during this time.

The results of the three experiments are given in Table II, the figures representing the immunity percentage 3 weeks after inoculation with cancer (Text-fig. 3).



TABLE II.

Experiment No.	Group 1.	Group 2.	Group 3.
7	66.6 per cent immunity.	40.0 per cent immunity.	20.0 per cent immunity.
8	55.0 " " "	35.0 " " "	10.0 " " "
9	50.0 " " "	30.0 " " "	10.0 " " "

Group 1 was made up of mice heated 1 week before inoculation. Group 2 mice were heated immediately before inoculation, and Group 3 comprised the control mice. The results are based on 118 mice.

EXPERIMENT 8									
Heated 7 days before inoculation, 55% immune			Heated immediately before inoculation, 35% immune			Normal controls, 10% immune			
1	-	-	-	-	-	+	-	-	
2	+	-	-	+	-	+	+	-	
3	-	-	-	-	-	-	-	-	
4	+	-	-	-	-	-	-	-	
5	-	-	-	-	-	-	-	-	
6	-	-	-	+	+	-	-	-	
7	+	-	-	-	-	-	-	-	
8	+	-	-	-	-	-	-	-	
9	+	-	-	-	-	-	-	-	
10	+	-	-	+	-	-	-	-	
11	+	-	-	-	-	-	-	-	
12	+	-	-	-	-	-	-	-	
13	+	-	-	-	-	-	-	-	
14	+	-	-	+	-	-	-	-	
15	+	-	-	-	-	-	-	-	
16	+	-	-	-	-	-	-	-	
17	+	-	-	+	+	-	-	-	
18	+	-	-	-	-	-	-	-	
19	+	-	-	-	-	-	-	-	
20	+	-	-	-	-	-	-	-	
Weeks 1	2	3	1	2	3	1	2	3	

TEXT-FIG. 3. The effect of dry heat on the immunity to transplanted cancer when exposure to heat was done 7 days and immediately before inoculation.

The foregoing experiments indicate a definitely higher resistance in the mice inoculated immediately after heating than in the controls, while the mice heated a week prior to inoculation show an even more pronounced degree of immunity. The blood count after the heat treatment showed a sharp but very transitory drop in the number of circulating lymphocytes, followed within 24 hours by a marked increase and a continuous rise in these cells lasting for a week or more. Histological examination of the lymphoid organs of heated

animals showed that by 48 hours after the treatment these organs contain a larger number of mitotic figures in the germinal centers than is normally seen. The height of the reaction, judging from the blood pictures and the condition of the spleen and lymph nodes, occurs about the 7th day after the exposure to the heat. The degree of immunity to the transplanted cancer seems to vary directly with the amount of stimulation of the lymphocytes existing at the time of or immediately following the inoculation.

#### SUMMARY.

It has been shown that resistance to transplanted cancer follows stimulation of the lymphoid tissue when the stimulation is induced by either heat or small doses of x-rays. In this paper we have attempted to determine whether the degree of immunity had a quantitative relation to the amount of the stimulation. Fortunately, the two methods at our disposal give stimulation of markedly different characters. The small dose of x-rays gives a sluggish lymphoid cell reaction of short duration with a definite latent period between the treatment and the evidence of marked stimulation, while after heat a short period of depression is followed by a sharp stimulation continuing over a much longer period. The cancer inoculation into groups of mice made immediately after exposure to x-rays shows little resistance, while the inoculation made at the height of the stimulation phase shows a definite increase in the immunity. Animals inoculated with cancer immediately after the heat treatment exhibit a pronounced immunity, but not so marked as that shown when the inoculation is made at the height of the stimulation.

The amount of resistance shown when the cancer inoculation is made at the height of the moderately stimulating effect following exposure to x-rays, is much less than that seen when the inoculation is made at the height of the heat effect when the degree of stimulation is much greater. When the lymphocytosis sets in after the tumor graft is established only a slight effect is noted. All these results together are taken to indicate that the degree of immunity is dependent on the amount of lymphoid stimulation existing either at the time of or following soon after the cancer inoculation.

## STUDIES ON X-RAY EFFECTS.

### VII. EFFECT OF SMALL DOSES OF X-RAYS OF LOW PENETRATION ON THE RESISTANCE OF MICE TO TRANSPLANTED CANCER.

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(Received for publication, October 22, 1920.)

Murphy and Morton<sup>1</sup> have shown that small doses of x-rays capable of stimulating somewhat the circulating lymphocytes increase the resistance to the growth of spontaneous tumors in mice. In their experiments the tumor was first removed by operation, the animal was treated with one small dose of x-rays, and the graft of the original tumor was returned to the left groin of the same animal, any direct effect of x-rays on the tumor being thus avoided. Studies of the stimulative effect of x-rays, generated by a Coolidge tube, on lymphoid cells showed that the dose of x-rays governed by the following factors will increase lymphoid activity in the rabbit:<sup>2</sup>  $\frac{7}{8}$  inch spark-gap, milliamperage 25, distance from target 8 inches, and time of exposure 20 minutes. This dose with its original factors was not satisfactory when applied to another species of animal, namely mice, but by shortening the time of exposure it was possible to induce an active proliferation of lymphoid tissue in these animals.<sup>3</sup> The best results were obtained when the exposure was of 10 minutes duration.<sup>4</sup>

There have been accumulated numerous facts which point to the conclusion that the activity of lymphoid cells is a factor governing resistance to the growth of cancer. With the determination of the x-ray dose which suffices to induce lymphoid stimulation, another opportunity was offered to test the part played by lymphoid cells in this state of resistance.

<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

<sup>2</sup> Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

<sup>3</sup> Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

<sup>4</sup> Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35.

## EXPERIMENTAL.

Young adult white mice were exposed to the following dose of x-rays: spark-gap  $\frac{7}{8}$  inch, milliamperage 25, distance from target to back of mouse in the ordinary attitude 8 inches, and time of exposure 10 minutes. From 3 to 7 days afterwards the mice, together with a suitable number of controls, were inoculated subcutaneously in the left groin with a bit of a transplantable cancer (Bashford Adenocarcinoma No. 63). The rate of growth of the tumor was noted at weekly intervals thereafter.

Table I gives the number of mice and the degree of resistance shown at the end of 3 weeks after inoculation (Text-figs. 1 to 3).

TABLE I.

Experiment No.	Interval between exposure to x-rays and tumor inoculation.	Immunity in x-rayed animals.	Immunity in controls.
	<i>days</i>		
1	3	40.0 per cent (10 mice).	11.1 per cent ( 9 mice).
2	5	50.0 " " (16 " ).	10.0 " " (20 " ).
3	5	20.0 " " (10 " ).	0.0 " " (10 " ).
4	7	75.0 " " ( 8 " ).	23.3 " " ( 9 " ).
5	7	10.0 " " (10 " ).	0.0 " " (10 " ).
6	7	30.0 " " (10 " ).	0.0 " " (10 " ).
7	7	71.4 " " (21 " ).	40.0 " " (10 " ).

During the course of these experiments the virulence of the tumor used varied considerably. In Experiments 3, 5, and 6 the controls showed no resistance, and correspondingly the immunity was low among the x-rayed animals. Attention is called particularly to Experiment 5 (Text-fig. 2) in which, at the end of 3 weeks, only one animal in the x-rayed series showed absolute immunity. A definite retarding effect was evident, however, in that at the end of the 1st week all but two of the controls showed definite tumors, while only three of the x-rayed animals proved to be susceptible. By the 2nd week after inoculation all the control mice had palpable tumors and only five of the x-rayed animals. This experiment is a very good illustration of how the more virulent tumors may break through a

resistance. While the degree of resistance induced by this dose of x-rays is low, yet it is consistently higher in the treated animals than in the controls, even with the varying growth energy of the tumors.

X-rayed 5 days before tumor inoculation				Controls		
1	-	•	•	-	-	•
2	+	•	•	-	-	•
3	-	•	•	-	-	•
4	-	•	•	-	-	•
5	-	+	•	-	-	•
6	-	-	•	+	•	•
7	•	+	•	•	•	•
8	+	•	•	+	•	•
9	+	-	-	+	-	•
10	•	-	-	-	•	•
11	•	-	-	+	•	•
12	•	-	-	•	•	•
13	+	-	-	+	•	•
14	+	-	-	+	-	•
15	+	-	-	+	•	•
16	•?	+	-	•	•	•
17				+	+	•
18				•	•	Died
19				•	•?	-
20				+	-	-
Weeks 1	2	3		1	2	3

TEXT-FIG. 1.

X-rayed 1 week before tumor inoculation				Controls		
1	•	•	•	-	•	•
2	•	-	•	•	•	•
3	•	•	•	•	•	•
4	+	•?	•	•	•	•
5	+	•	Died	•	•	•
6	+	•	•	•	•	•
7	+	-?	•	•	•	•
8	-	-	•	•	•	•
9	+	-	•	+	•	•
10	+	-	-	+	•	•
Weeks 1	2	3		1	2	3

TEXT-FIG. 2.

TEXT-FIG. 1. Experiment 2. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

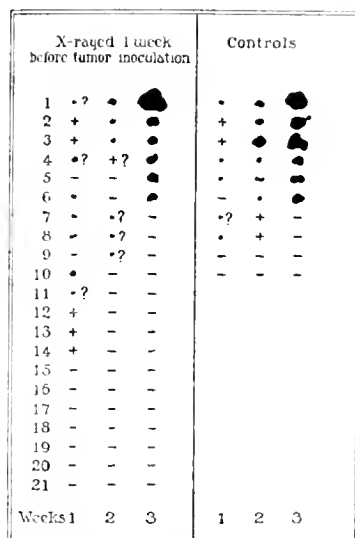
TEXT-FIG. 2. Experiment 5. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

#### DISCUSSION.

The foregoing experiments show conclusively that x-rays given in a dose sufficient to stimulate the lymphoid tissues, increase the resistance of mice to a transplanted cancer. As the degree of stimulation induced by this agent is less than that induced by exposure to intense dry heat or that following the injection of homologous living tissue, it is not surprising that the degree of resistance is less than that seen after the employment of the latter two methods.

It is of interest to note that the degree of resistance exhibited by the x-rayed mice varied with the growth energy of the tumor as shown by the number of takes in the control animals.

Our original observation that x-rays in small amounts increased the resistance of a mouse to transplants of tumors,<sup>1</sup> was later confirmed by Russ, Chambers, Scott, and Mottram,<sup>5</sup> who used transplants of the Jensen rat sarcoma with rats as their subjects. They gave an exposure of 12 seconds repeatedly, instead of the single dose which we have used.



TEXT-FIG. 3. Experiment 7. The rate of growth of Bashford Adenocarcinoma No. 63 in mice x-rayed before inoculation, contrasted with the rate of growth in untreated mice.

#### SUMMARY.

A relatively increased degree of resistance in mice to a certain strain of transplantable cancer was demonstrated after treatment of animals with small doses of x-rays capable of stimulating lymphoid tissue. The refractory state induced was determinable 3 to 7 days after the dose of x-rays was given.

<sup>5</sup> Russ, S., Chambers, H., Scott, G. M., and Mottram, J. C., *Lancet*, 1919. i. 692.

## STUDIES ON X-RAY EFFECTS.

### VIII. INFLUENCE OF CANCER INOCULATION ON THE LYMPHOID STIMULATION INDUCED BY SMALL DOSES OF X-RAYS.

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(Received for publication, December 31, 1920.)

The studies carried out in this laboratory have shown that immunity to cancer, whether natural or induced, is attended by lymphoid stimulation.<sup>1</sup> X-rays have been employed to secure the stimulative effect on the lymphoid elements;<sup>2</sup> when of sufficient amount, they have been found to increase<sup>3</sup> the resistance of mice to cancer transplants<sup>4</sup> made at the height of the reaction. On the other hand, if the cancer inoculation is made immediately after the x-ray treatment, no unusual degree of resistance is exhibited.<sup>5</sup> Hence it would appear that the early inoculation of cancer must have in some way interfered with the development of the defensive mechanism. The present study has been planned to elucidate this point.

#### EXPERIMENTAL.

*Experiment 1.*—Twelve normal white mice were given the following dose of x-rays (Coolidge tube): spark-gap  $\frac{7}{8}$  inch, milliamperage 25, distance 8 inches, and time of exposure 10 minutes. The manner of applying the dose was the same as in experiments previously reported. As soon after the treatment as possible the animals were inoculated subcutaneously in the left groin with fragments of a Bashford adenocarcinoma, No. 63. Of these mice, six were killed for histo-

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<sup>1</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

<sup>2</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

<sup>3</sup> Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

<sup>4</sup> Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1921, xxxiii, 429.

<sup>5</sup> Murphy, Jas. B., *Proc. Nat. Acad. Sc.*, 1920, vi, 35. Murphy, Jas. B., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 423.

logical study 48 hours and the other six 4 days after the inoculation. No difference was noted at autopsy between the two groups of mice killed at different periods.

In three of the six animals in each group the spleen and lymph nodes were found at autopsy to be smaller than is usual in normal mice. The mesenteric node, which is the largest lymph node in the mouse, was in a few instances as small as the normal inguinal, or even the axillary node.

Microscopically, a considerable number of pycnotic cells were found in these organs, but the deposit of pigment in the spleen was never conspicuous. The most striking feature was the almost complete suppression of the proliferative activity of lymphoid cells in half of the animals examined, only a few mitotic figures being found in each section. This suppressed activity was equally evident in the two groups, and apparently was independent of the size of lymphoid organs. In the remaining half of the animals, mitotic figures were found more frequently, but in no instance was there any sign of an activity above normal.

*Experiment 2.*—Eleven normal white mice were given the same dose of x-rays as before; six were killed 48 hours and five 4 days after the treatment, without having been inoculated with the cancer.<sup>6</sup>

The increase in the number of mitotic figures in the lymphoid tissue was evident in the majority of cases; namely, in nine out of eleven animals. The remaining two animals showed only slight signs of lymphoid proliferation, judging by the limited number of mitotic figures found.

*Experiment 3.*—Eleven normal white mice were treated with the same dose of x-rays. 7 days later the animals were inoculated subcutaneously in the left groin with fragments of Bashford Adenocarcinoma No. 63. Six of these mice were killed after 48 hours and five after 4 days; *i.e.*, 9 and 11 days after the x-ray treatment.

No unusual macroscopic feature of the lymphoid organs was noted at autopsy. There was some variation in the histologic condition of these organs. In six of the eleven animals, there was an extensive stimulation of lymphoid tissue as evidenced by the large number of mitotic figures in the tissue. The mitotic figures were found abundantly, not only in the area of the germ centers, but also in the pulp. The nodules were more or less enlarged. There were few pycnotic cells, and pigment was almost entirely absent. In four other animals, the stimulative reaction was similar in kind but somewhat less pronounced. One animal was exceptional in that it showed almost no signs of cell proliferation.

*Experiment 4.*—Six normal white mice were treated with the same dose of x-rays as those above but were not inoculated with cancer. Three were killed 9 days and three 11 days afterwards.

The spleen of four out of the six mice was found at autopsy to be abnormally dark in color and, except in one animal in which it was below normal, of usual size.

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<sup>6</sup> This was a repetition, for the sake of comparison, of an experiment previously reported.<sup>3</sup>



Histologically, the dark spleens showed a great accumulation of blood in the pulp, which was sparingly supplied with lymphoid cells. In contrast to the findings in Experiment 3, mitotic figures were in no instance abundant in the lymphoid tissue of spleen or of the lymph nodes. Cells with pycnotic nuclei were numerous, except in one specimen in which the number was small.

For a further comparison, the four preceding experiments were repeated in a single experiment which included groups subjected to the various experimental conditions of the individual experiments described above.

*Experiment 5.*—Twenty-five normal white mice, divided into four groups, were given the same dose of x-rays as in the previous experiments.

Group 1: eight mice were inoculated with Bashford Adenocarcinoma No. 63 immediately after the treatment with x-rays. They were killed 3 days later.

Group 2: seven mice were killed 3 days after the x-ray treatment without having been inoculated with cancer.

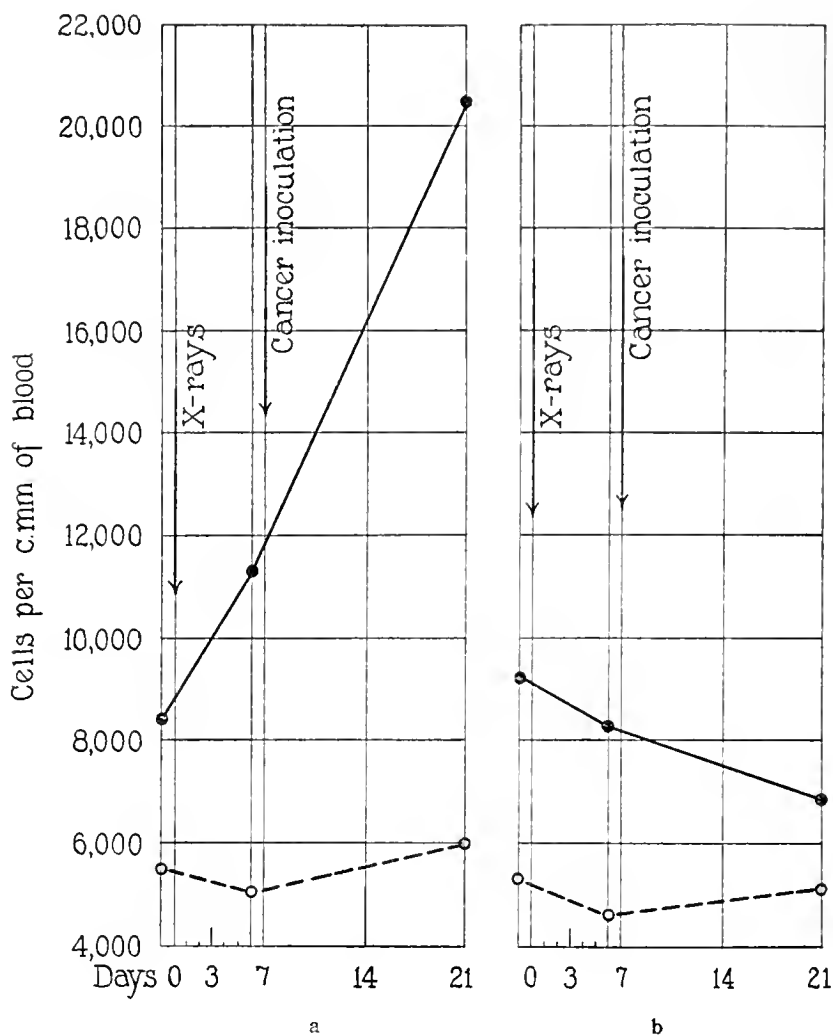
Group 3: six mice were inoculated, 7 days after the treatment with x-rays, with the same strain of cancer and were killed 3 days after the inoculation; *i.e.*, 10 days after the x-ray treatment.

Group 4: four mice uninoculated with cancer were killed 10 days after the x-ray treatment.

Of Group 1, all the mice except one individual showed suppression of the usual proliferation of the lymphoid elements. Of Group 2, all showed definite signs of increased proliferation of the same elements. In Group 3, three mice showed evidences of extensive stimulation, while the remaining three had a less marked reaction. Group 4 showed little signs of lymphoid proliferation.

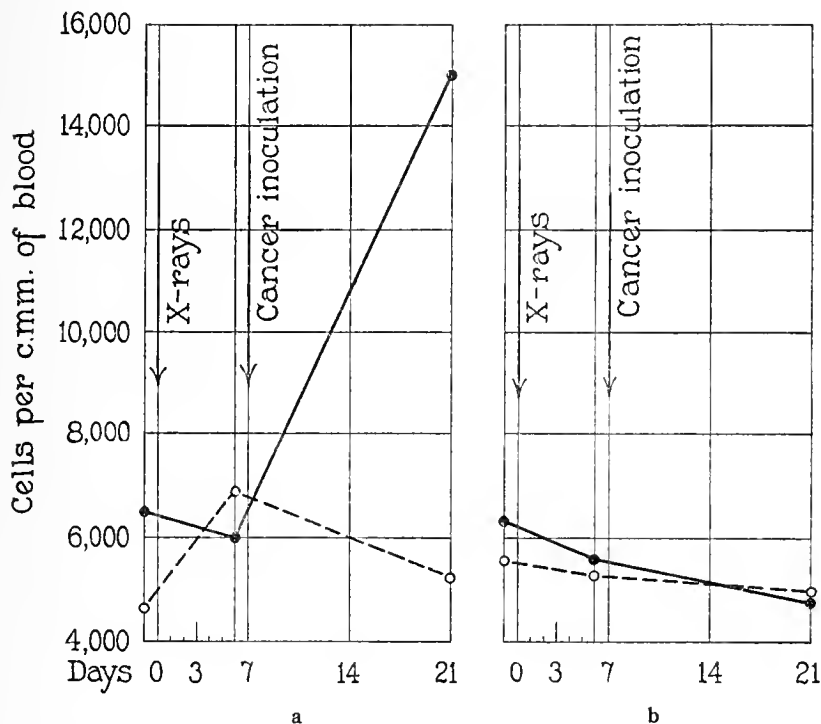
Hence, the results of this experiment are in agreement with those of the four experiments immediately preceding.

The following conclusions can be deduced from the results. (1) Cancer inoculation made immediately after a stimulative dose of x-rays interferes with the lymphoid reaction and little or no stimulation results. (2) Cancer inoculation made at the height of the stimulation augments the lymphoid reaction, and the proliferative activity of the cells continues longer than in animals which have been stimulated but have not received the cancer inoculation. (3) A proportion of animals given the stimulative dose of x-rays fails to react. It is of considerable interest to ascertain whether mice which fail to react are susceptible to cancer inoculations. To determine this point we have used the blood counts to ascertain the absence or presence of a stimulative phase.



TEXT-FIG. 1, *a* and *b*. Experiment 6. Composite curves of white blood cell counts on mice x-rayed and inoculated with cancer 7 days later. (*a*) Composite curves from fourteen mice proved to be immune. (*b*) Composite curves from ten mice proved to be susceptible. — Lymphocytes. - - - Polymorphonuclear leucocytes.

*Experiment 6.*—Blood counts were made on twenty-four mice which were then given an exposure of x-rays similar to that used in the preceding experiments. 7 days later a second count was made, followed by the inoculation of each animal with a graft of the Bashford mouse cancer, and 14 days later a third count. Fourteen of the twenty-four animals resisted and ten responded to the cancer inoculation.



TEXT-FIG. 2, *a* and *b*. Experiment 7. Composite white blood cell counts on mice x-rayed and inoculated with cancer 7 days later. (*a*) Composite curves from the immune mice. (*b*) Composite curves from the susceptible mice. ———— Lymphocytes. - - - - - Polymorphonuclear leucocytes.

In the immune mice (Text-fig. 1, *a*) the average number of lymphocytes per c. mm. of blood before x-ray treatment was about 8,300, the number of polymorphonuclear leucocytes being about 5,400. 6 days after the treatment (1 day before cancer inoculation) lymphocytes and polymorphonuclear cells were about 11,400 and 5,000 respectively. 2 weeks after the cancer inoculation the lymphocytes had, however, risen to approximately 20,000, while the polymorphonuclear cells showed but little change, being about 6,000.

In susceptible mice (Text-fig. 1, *b*) the average number of lymphocytes per c. mm. of blood before the dose of x-rays was approximately 9,600, the average number of polymorphonuclear cells was 5,300. A slight decrease in the white cells was noted 6 days after the treatment, the lymphocyte and polymorphonuclear leucocyte counts being 8,000 and 4,600 respectively. 2 weeks after the cancer inoculation the lymphocyte count was 7,000, the polymorphonuclear cell count 5,000.

*Experiment 7.*—The preceding experiment was repeated with twenty-six x-rayed mice, only nine of which resisted the cancer inoculation. Blood counts were made in the same way as before.

The average number of lymphocytes per c. mm. of blood in immune mice (Text-fig. 2, *a*) before x-ray treatment was about 6,500, and of polymorphonuclear leucocytes about 4,500. 6 days after the x-ray treatment the lymphocytes showed no material change, but the polymorphonuclear leucocytes went up to about 6,500. 2 weeks after the inoculation of cancer there was a rise of lymphocytes to about 15,000, while the polymorphonuclears decreased to about 5,000.

In susceptible mice (Text-fig. 2, *b*) the average number of lymphocytes and polymorphonuclear cells per c. mm. of blood before exposure to x-rays was about 6,000 and 5,000 respectively. No material change in these numbers was observed 6 days after x-rays. 2 weeks after cancer inoculation, however, the lymphocytes and polymorphonuclear cells were slightly decreased in number, the former being about 4,000 and the latter about 4,500.

#### DISCUSSION.

A comparison of the experiments described leads to the conclusion that if cancer inoculation is made immediately after the stimulative treatment with x-rays, no lymphoid stimulation occurs such as would regularly occur if the cancer inoculation were not made. On the other hand, if cancer inoculation is made 7 days after the x-rays are given, thus allowing the stimulation to develop before the inoculation, there is in the majority of cases what might be called a second stimulation of lymphoid tissue. It is significant, in connection with these facts, that while only little resistance to the transplantation was discovered when cancer was inoculated immediately after the x-rays, evidence of increased resistance appeared when inoculation was postponed until the 7th day.<sup>5</sup>

Attention is drawn in this connection to the parallelism existing between the lymphoid reaction accompanying the immunity to cancer grafts induced by physical agents (x-rays) and that induced by a biological agent (homologous blood). We have already shown

that mice immunized to cancer by means of an injection of defibrinated blood show an increase in the number of mitotic figures in the lymphoid tissue. Such mice, when inoculated with a cancer graft 10 days after the injection exhibit a second stimulation of the tissue,<sup>7</sup> as well as a marked blood lymphocytosis.<sup>1</sup> The experiments reported indicate a corresponding effect brought about by small doses of x-rays. The blood counts on the animals after the cancer inoculation show that only the animals presenting an increase in the lymphocytes prove to be resistant to the cancer. However, it should be stated that the blood counts were not made at a time to show the primary stimulative effect of x-rays, since this reaction, as previously shown, is of short duration. Hence it appears that as a result of the primary stimulation the animals have acquired the ability to react more strongly to a second stimulation; namely, the cancer inoculation.

#### SUMMARY.

Mice treated with small doses of x-rays and inoculated with cancer immediately afterwards, show a marked suppression of lymphoid proliferation. If, however, the cancer inoculation is made 7 days after the exposure to x-rays, thus permitting the primary lymphoid stimulation known to occur soon after the x-ray treatment to arise, a second stimulation takes place in a large proportion of mice thus inoculated.

Changes in the blood of mice x-rayed and inoculated with cancer 7 days afterwards show that the state of resistance to cancer inoculation is attended by blood lymphocytosis, as is the case in all other varieties of immunity to transplanted cancer so far studied.

<sup>7</sup> Murphy, Jas. B., and Nakahara, W., *J. Exp. Med.*, 1920, xxxi, 1.



# THE ETIOLOGICAL RELATION OF BACILLUS ACTINOIDES TO BRONCHOPNEUMONIA IN CALVES.

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PLATES 42 TO 50.

(Received for publication, December 28, 1920.)

In the early months of 1917 an outbreak of pneumonia occurred among calves belonging to a large dairy herd in which about 100 calves are raised annually. Up to May 1, eleven cases had been autopsied. Nine of these were killed in advanced stages of the disease and two died. Scattering cases occurred at the rate of about one a month into November. Of these, one died and seven were killed. In the course of the investigations a minute bacillus was isolated whose etiological relation to the pneumonia was left in doubt.<sup>1</sup> In this outbreak a peculiar disease of the kidneys occurred in ten out of the nineteen cases. The lesions were situated in the cortex and consisted of foci of sclerosis with destruction of the secreting tissue.

More than 2 years later, in October, 1919, there began in the young stock of the same herd a similar series of cases. Up to March of 1920, ten cases came to autopsy. Of these four had died and six were killed. The kidney lesions were absent. A belated case was observed in June. There may have been more cases which recovered without being detected, since no careful clinical examination was made and the disease was recognized only when the respiration had become sufficiently abnormal to warrant removal from the herd. The ratio of animals openly diseased to the entire number of calves was relatively low. This may have been due to the fact that the calves were kept in groups of six to eight in adjoining open pens, those of nearly the same age being kept together.

<sup>1</sup> Smith, T., *J. Exp. Med.*, 1918, xxviii, 333

In the second outbreak the lesions of the respiratory tract, more particularly of the lungs, were the chief if not the only ones. In the first, as stated above, a nephritis was associated with over half the cases. In the present article references to the nephritis will be omitted and made the subject of a separate paper. A description of the bacillus was published in 1918.<sup>1</sup> Its chief distinguishing characters are briefly as follows:

1. Multiplication in the form of small flakes up to 1 mm. in diameter which consist of parallel bundles of filaments, each filament terminating in two club-like expansions, one at each end. The flake thereby becomes more or less rounded, mulberry-like, and resembles a sphere with clubs projecting from the central mass. In this *Actinomyces*-like growth and within the sheathing filaments are chains of minute bacilli.

2. The sheaths and clubs are produced in the condensation water of coagulated serum and to a less extent on the sloped surface but not on agar plus tissue or blood. On these latter media the growth is feeble and the organism appears in the form of rods without the sheaths. It often fails entirely.

3. On ordinary media such as plain agar and bouillon the bacillus multiplies very faintly or not at all.

4. After 10 or more days growth on the agar plus blood or tissue, minute refringent bodies are found free in large numbers and also within the remaining rods. In some cultures all bacilli had disappeared. This phenomenon has been tentatively interpreted as spore formation.

5. The bacillus fails to produce any appreciable lesions in small animals.

#### *Pathological Anatomy and Bacteriology of Individual Cases.*

The gross appearances of the lungs differed very much from animal to animal but the cases could be grouped according to certain characters into acute, chronic, and intermediate types. The important features of the pneumonia, both pathological and bacteriological, are best presented by a brief description of certain cases which will form the basis for a discussion of the etiology. For the names applied to the different lobes of the cow's lungs in these pages, the reader is referred to the description of Text-figs. 1 and 2, page 450.

*No. 440.*—Female calf, aged 49 days. Died suddenly Nov. 12, 1919, and was autopsied within a few hours. There is no history of any pronounced illness preceding death, although this may have been overlooked by the attendant.

The lesions are restricted to the respiratory organs. The lungs are affected symmetrically. Both cephalic and ventral lobes are solidified with the exception



of six or more small air-containing regions along caudal border of right cephalic lobe and several along dorsal margin of the same lobe. Near root of right ventral lobe there is an air-containing region several centimeters in diameter. The large caudal lobes are solidified in the cephalic third or half. The involved tissue covers much more of the diaphragmatic than of the dorsal surface of these lobes. Air-containing lobules encroach on the hepatized territories and foci of collapsed lobules extend into and permeate the air-containing portions. The small azygous lobe consists of inflated and dark red collapsed regions intermingled.

The involved lobes are as firm as liver tissue to the touch. Their dimensions are about twice those of lungs normally collapsed. The gross appearance of the pneumonic regions differs from lobe to lobe. The cephalic and ventral lobes show closely set, grayish dots 1 to 3 mm. in diameter on a dark red ground, excepting a strip comprising the dorsal border, where the tissue is uniformly dark red, lacking the lighter dots. Similarly, the caudal lobes show the mottled character in a strip adjacent to the ventrals and the smooth, more uniform condition caudally, bordering on the still air-containing regions of these lobes. The mottling is also found on section. Embedded in one ventral lobe there are several foci, 4 to 5 mm. in diameter, which are made up of a semisolid caseous matter. Quite small foci 1 to 2 mm. in diameter of similar character are found here and there in the other lobes.

Larynx and trachea are normal. At bifurcation there is a mass of whitish mucoid substance. Both bronchi are deeply congested and flecks of whitish, soft, mucoid material are scattered over the mucosa. When the lobes are cut across and gently compressed, minute whitish molds are forced out of the smaller bronchioles. The lymph nodes, both mediastinal and bronchial, are somewhat enlarged, on section slightly congested and juicy. Occasional whitish, point-like foci are seen on the cut surface. The pleura is normal. The heart is flabby; pericardium normal. In the fat around the base of the right ventricle there is some patchy hemorrhage. Both sides of the heart contain large, dark, soft clots extending as cylindrical molds into all the large vessels.

Sections from fixed and hardened tissue of the various lobes stained in eosin and methylene blue present the following features. The small air tubes are filled with a mixture of cells and a peculiar, faintly bluish red, homogeneous material, probably mucus. The relative amount of this and of cellular material varies from tube to tube. Within the parenchyma there are foci consisting each of a group of alveoli which are packed with cell masses and the homogeneous substance referred to above. Among the desquamated alveolar epithelial cells are smaller cells the nuclei of which have contracted into irregular star-shaped, solidly stained masses of chromatin or into other irregular shapes, simulating the nuclei of polynuclear leucocytes. They are either lymphocytes or endothelial cells. A few polynuclear leucocytes permeate the mass of cells and debris. The cell foci may be so numerous as to coalesce, or there may be between them a zone of alveoli in which only a few desquamated alveolar cells are seen. It is these cell foci which appear to the naked eye as the grayish subpleural dots. The cell masses in the bronchioles and the alveoli are the same in character.

Another feature of significance is the presence within the smallest air tubes of what appear to be ingrowths from the wall (Figs. 1 to 3). These ingrowths consist of cells of the types described as present in the alveoli, embedded in a feebly stained matrix. The epithelium is missing where the ingrowth takes place. Within this matrix are dense masses of bodies varying in shape from minute rods in small colonies to groups of roundish bodies 2 microns in diameter with only the outline stained (Figs. 4 to 7). These latter bodies are also found in the alveolar cell masses but much less abundantly than colonies of minute bacilli (Figs. 8 and 9).

The origin of the cloud-like masses of bacteria pushing into the lumen of the air tube is obscure. A prolonged examination of sections from this and other cases makes it probable that the bacteria start in the cytoplasm of the epithelial cells, develop into colonies, and thereby cause a great increase in the size of the cells and their eventual destruction. The mass thus projects into the lumen where some of the migrating cells fuse with it, producing the characteristic obstructing plug. It is doubtful whether there is any actual hyperplasia of the epithelial cells. In the cell foci of the parenchyma a similar invasion of the alveolar cells takes place, leading to destruction of such cells and the appearance of hemorrhages and of the lymphocyte cell masses.

Cultures were made on slanted coagulated horse serum and slanted agar with and without a few drops of horse blood. All tubes were hermetically sealed and incubated. The cultures were made both by transferring bits of lung tissue and by inoculating the tubes with a heavy platinum wire thrust into the pneumonic tissue. After several days, growth appeared and in all but a few tubes *B. actinoides* was present in pure culture. In the remaining tubes a few foreign colonies were also present.

No. 450.—Black and white female calf. Born Oct. 27, 1919. Attendant reported that on Nov. 27 the calf did not eat its food and that breathing was rapid. From that day on the temperature was taken several times and found to be around 40.5°C. There was much coughing, the respirations continued short and superficial, and emaciation progressive. Dec. 4. The calf, then weighing 85 pounds, was killed by stunning with a heavy blow, clamping the trachea to prevent aspiration of stomach contents into the lungs, and opening the vessels of the neck. There was nothing noteworthy about the organs excepting the respiratory tract.

When the thorax was opened the lungs collapsed with force. The distribution of the pneumonic lesions is somewhat less extensive than in the preceding case. Both cephalics and ventrals are entirely consolidated. The cephalic half of the azygous lobe and a narrow strip along the cephalic margin of the right caudal lobe are involved. The left caudal lobe is intact. There is a narrow margin of air-containing lung tissue along the dorsal ridge of both lungs almost to the cephalic tip, also small inflated lobules in the left ventrocephalic lobe.

The condition of the consolidated lobes is not the same throughout. The right cephalic and the right ventral lobes are much larger than the normal collapsed

condition would be. They are grayish red to grayish yellow in appearance. The remainder of the affected lung is but a trifle larger than in the normal collapsed state and dark red with a faint, regular mottling of a lighter color. In this lung the grayish dots or granulations characteristic of the preceding case are absent. The pleura is normal.

The lower portion of the trachea and the main bronchi at the bifurcation contain masses of a viscid, opaque, whitish, dough-like mucopus. The same glairy, pearly white masses can be expressed from the cut ends of the minute air tubes of the dark red lobes. Only a small amount is expressible from the air tubes of the large grayish lobes. This glairy matter is very tenacious and breaks up into fragments when crushed between cover-glasses. The bulk of the material is made up of polynuclear leucocytes. The dark reddish tissue is quite moist when rubbed on covers, the grayish less so. The latter leaves on the cover-slip a smeary, milky film. Projecting from the right ventral lobe is a whitish mass the size of a pea. It is made up of a thin walled cyst filled with a readily dislodged, dough-like mass. Cover-glass films show abundant alveolar epithelium, polynuclear leucocytes, and several dense masses of very fine, rather feebly stained bacilli. Films from tracheal and bronchial mucopus and from lung parenchyma contain the same cellular and bacterial elements.

The difference between the gross pictures of the lungs of Nos. 440 and 450 is paralleled by differences in the microscopic picture. The peculiar cell infiltration in No. 440 has disappeared and polynuclear leucocytes have taken its place. The cell plugs in the air tubes contain chiefly polynuclears among which are scattering alveolar cells. The peculiar amorphous, homogeneous material which was regularly associated with colonies of bacteria in No. 440 is absent and bacteria are not detected. The parenchyma is the seat of polynuclear infiltration in foci which coalesce in the older stages and in the more recent stages gather irregularly in the collapsed tissue. The invasion of new territories is characterized by a ring of fibrin-blocked alveoli around the invaded air tube and occasionally hemorrhages. The interlobular tissue contains oval masses of fibrin lodged in the distended lymph spaces.

The difference between the two cases is further indicated by a new feature. All small air tubes are surrounded by a loose zone of cells which resemble plasma cells in form and the basophilic character of the cytoplasm and which collect in the subepithelial tissue.

Cultures prepared from the different lobes by transferring bits of lung tissue to horse serum, agar and blood, and plain agar, as well as by inoculating the same kind of media with heavy and fine platinum wire forced into the lung tissue yielded cultures of *B. actinoides* which were pure with the exception of those from the azygous lobe in which a few large streptothrix-like colonies appeared. The tubes inoculated with the heavy platinum wire developed, whereas most of those inoculated with a fine wire remained sterile.

*No. 446.*—This is a case which duplicates in all particulars No. 450 (Fig. 14). This calf was born in September and was 2 months and 2 days old when killed.

Cultures prepared from the different lobes in the manner described for No. 440 developed, with few exceptions, growths of *B. actinoides*. A few stray colonies of other kinds were present in several tubes.

No. 432.—This case may be classed as intermediate between the first and the following group. Female, born June 21; killed Oct. 21, when 4 months old. The meager data indicate that it was sick with some respiratory affection late in July and early in August. Early in October the respiration became short and quick, there was frequent coughing, and the calf breathed with mouth open. The respirations continued short and labored until it was killed. The temperature fluctuated between 39° and 40°C. The animal was killed by a stunning blow on the head, clamping trachea, and severing vessels of neck.

The consolidation of the lungs is of the usual extent. The pneumonic process involving completely the cephalic half of both lungs has invaded both caudal lobes and is separated from the air-containing tissue by a sharp, irregular, jagged line. Pneumonic lobules also appear as islands in these lobes. The trachea contains whitish, mucoid masses and the small air tubes of the affected lobes are filled with molds of similar material.

The hepatization is very firm, the affected lobes about twice the dimensions of the normal collapsed state. The surface is mottled like that in No. 440. Necrosis is absent. The cut surface shows the walls of the small air tubes distinctly thickened. In general the gross appearance indicates that the process is more or less of the same age throughout, except in the caudal lobes, where it is freshest. The mucopurulent molds from the small air tubes and the loose masses in the trachea contain large numbers of very minute and somewhat larger bacilli. Histological examination of sections from the different lobes indicates a transition in the pathological process from that of No. 440 to that of No. 450. In some regions the infiltrating cell masses are mononuclear, the contents of the minute air tubes largely made up of the homogeneous, bluish stained substance and groups of minute bacilli. In others, polynuclears predominate both in the parenchyma and the air tubes, and bacteria are rare or absent.

The bacteriology of the processes in this lung is complicated. On the agar and blood agar slants many roundish, rather fleshy colonies develop which have the cultural characters of the hemorrhagic septicemia group but with very low virulence. The general presence of *B. pyogenes* is indicated by liquefying colonies in all horse serum tubes. A third type of bacilli not identified was present in some tubes. Identifiable by its peculiar growth, *B. actinoides* was demonstrated as occurring in most tubes, but repeated attempts to obtain it in pure culture failed, owing to the predominance of the other types.

A type of pneumonia more acute than that represented by No. 440 was observed in three cases. No. 429, the first of the present outbreak to come to autopsy, was born September 7 and died October 12. The second, No. 436, was born September 20 and died

November 4. The third, No. 447, was born October 17 and died November 29. The three calves were thus respectively 35, 45, and 43 days old at the time of death. The three cases present certain differences among themselves.

*No. 429.*—All lobes are much enlarged over the normal collapsed size. There is an adhesion of most of cephalic and ventral lobes to ribs and to pericardium. The attachments are easily broken except the adhesion of right cephalic lobe to pericardium which is not separable. Consolidation of all but one-half of right caudal and two-thirds of left caudal lobe. There is a general faint putrefactive odor emanating from the lungs. The affected lung tissue is dark red and sprinkled over with numerous yellow, cheesy foci 2 to 5 mm. in diameter and projecting slightly above the pleura. These foci permeate the lung tissue with the exception of the caudal and azygous lobes in which such foci are few and small. Besides the small foci the right cephalic lobe contains a sequestrum 2 by 2 by 5 cm., yellowish, and firm like rubber. The trachea is uniformly reddened and covered with whitish, viscid flakes.

*No. 436.*—Extent of lung involvement and size of affected lobes are as in No. 429. The lung tissue resembles that of No. 440. It is sprinkled over densely with lighter grayish foci about 0.5 mm. in diameter. On pressure very little fluid and only a few consistent molds can be expressed from the air tubes. The multiple necroses found in No. 429 are absent. The mucosa of the trachea is uniformly congested, the bronchi deeply so, bordering on hemorrhage. The mucosa has on it some very thin patches of a pseudomembrane.

*No. 447.*—The involvement is somewhat less extensive than in Nos. 429 and 436. The pneumonic lobes are firm, only moderately larger than the normal collapsed state, uniformly dark red, and interspersed with a few inflated lobules. There is no distinct mottling with lighter dots as in No. 436, but the surface is rather variegated with larger patches of lighter color. Necrotic foci are not found. The trachea is clean and normal to the bifurcation where some flour-paste-like masses of mucopus are lodged. The main bronchi of affected lobes are filled with a similar thick pasty mass but those of the caudal lobes are free.

The microscopic picture in these three cases as constructed from sections of the different lobes is very much like that of No. 440 already described. The consolidation is due to focal infiltration of the parenchyma with the type of cell described under No. 440. Polynuclears are scarce or absent. The minute air tubes contain, besides cell masses, the homogeneous, bluish stained material, and it is in and among this material that masses of minute bacteria occur. The necroses of No. 429 occupy variable areas of lung tissue in which the alveoli have become impacted with the cell type mentioned. In these cases the gathering of plasma cells in the mucous membrane of the air tubes had not yet begun.

Cultures made from different parts of the lungs of the three cases indicated the presence of various species. Present in all three was *B. pyogenes*, especially abun-

dant in No. 429, less so in No. 436, and least so in No. 447. The sections containing the necrotic foci of No. 429 stained according to Gram-Weigert showed large, deep blue spots resolved as minute bacilli and evidently colonies of *B. pyogenes*. Several other types of colonies were found in the cultures. *B. actinoides* could not be detected in cultures from Nos. 429 and 436. Several cultures from No. 447 contained large numbers of colonies of *B. actinoides* but pure cultures failed because of the presence of *B. pyogenes*. Inasmuch as the type of disease was the same in the three cases, it is probable that *B. pyogenes*, being an acid producer, interfered with the development of *B. actinoides*.

Another group of cases is represented by calves in which the disease processes have gone on more slowly and to a much farther stage. The involved tissues have become necrotic and encapsuled, causing firm adhesions of the pleura to the chest wall and pericardium. There is a broad zone of plasma-like cells around the smaller air tubes, varying in size with the age of the calf.

*Case 455.*—Female calf born July 26; 4 months old lacking 3 days when killed. The attendant reported that this calf was sick with scours in August. Late in September it had a cold and in November symptoms of pneumonia were present. When killed it was very thin, the respirations short and labored. The cough was frequent, the temperature 40°C.

In this case also the pathological changes were limited to the respiratory organs. When the sternum was removed, the lungs collapsed vigorously. The pneumonic process had the usual extent and symmetrical distribution. The appearance of the lobes was much the same. The tissue is a reddish yellow and beset with closely crowded grayish areas, about 2 mm. in diameter, although varying more or less in size. Similar areas are seen on the cut surface. In the right cephalic lobe the grayish areas are larger and tend to coalesce. In the free tip of this lobe there is a group of larger foci about pea size, made up of thin walled sacs containing what appears to be in part necrotic lung tissue, in part mucus and pus. The pleura covering these foci gives rise to delicate fibers attaching lobe to pericardium. The mucosa of the trachea is normal and has lying in it white, curdy, viscid masses 4 to 5 mm. in diameter made up of polynuclear cells, alveolar epithelium, mucus, and some minute bacilli. All the small air tubes in the consolidated lobes when compressed exude thick, glairy, pearly white masses. Microscopically this material is like that in the lower trachea. All thoracic lymph nodes are distinctly larger than normal; on section quite moist and exuding a milky fluid.

The histological picture differs in degree from that of the more acute types. The most striking character is the presence of a zone of plasma cells under the epithelium of the air tubes, varying in thickness and especially broad in some lobes. The smallest air tubes are distorted, or else nearly obliterated so that their presence is only indicated by the plasma cell groups and zones. These cell zones plus

a broadening of the submucous layer probably represent the grayish areas seen from the surface and on section. The lung tissue around and between these zones is either slightly inflated and contains some desquamated cells, or else is collapsed or filled with polynuclear leucocytes. In sections bacteria are not seen, except in one large mass growing into and nearly obliterating the already dilated and deformed tube. In this mass colonies of fine, rather feebly stained bacilli are present. The flora of the pneumonic lobes of this case resembles somewhat that of No. 432. Of the large number of cultures, about thirty in all prepared with minute and pea size bits of lung tissue, all showed development. The colonies in each tube were few in number. *B. pyogenes* predominated in being present in most tubes. A second form, consisting of 2 to 3 mm., smooth, grayish, translucent colonies which tended to flow down the slant, was probably a variety of *B. bovisepiticus*. Several other forms were present. *B. actinoides* was detected in only two cultures among other forms. Among several cultures from bits of tissue from thoracic lymph nodes, one contained *B. pyogenes*.

*Case 462 (Text-Figs. 1 and 2 and Fig. 12).*—Female, born Nov. 4; killed Jan. 7, weighing at this time 102 pounds. It began life with scours which persisted for some weeks. Evidences of respiratory trouble began to appear Dec. 27. Although only a few days over 2 months old when killed, it presented advanced destructive lesions in larger number than any other case of this small outbreak. The pathological changes, if we except the general disappearance of fat deposits, were restricted to the lungs. Both cephalic and ventral lobes and a small adjacent zone of both caudal lobes were involved. The smaller lobes were adherent to the chest wall and pericardium through thin bands severed with some difficulty. In all but the caudal lobes are isolated or agglomerated, whitish, projecting nodes 5 to 10 mm. in diameter. The lung tissue between them is dark red and beset with paler grayish areas 2 to 3 mm. in diameter. The nodes are sacs containing a glistening, pearly white, viscid, flour-paste-like mass, rather sticky and not easily spread on cover-slips. It consists of cellular elements among which are dense masses of minute bacilli. Within this mucoid, whitish coating is a nucleus consisting of a lobulated mass not so white as the mucoid covering, more brittle, and finely spongy. The gross and microscopic characters indicate necrotic lung tissue. The walls of the cavities containing the sequestra are 1 to 1.5 mm. thick, and smooth. Where a number of cavities are close together they evidently communicate, since pressure continues to force the necrotic and mucoid masses out of all the cavities through one opening. Stained films of tracheal mucus and pus from the necrotic foci show besides cellular elements many minute bacilli frequently in dense masses. The air tubes of the affected lobes contain the same viscid, mucoid, purulent masses found in preceding cases.

The histological changes found in the various lobes do not differ materially from those of the advanced, chronic type. In general there is much collapse of the parenchyma with marked broadening of the alveolar walls due to infiltration of endothelial cell types. The collapsed tissue is focally occupied by dense infiltrations of polynuclear cells into the alveoli. In some lobes the normally distended

alveoli contain sparse collections of desquamated cells and polynuclears. The air tubes in some lobules have a dense enveloping zone of plasma cells. In others it is very slight. Sections through the necrotic foci show sequestra of lung tissue enclosed in a dense zone of plasma cells and more or less connective tissue stroma. Bacteria colonies are not seen.



TEXT-FIG. 1.



TEXT-FIG. 2.

Diagrams of involved lung tissue, Calf 462.

TEXT-FIG. 1. Dorsal aspect.

TEXT-FIG. 2. Ventral or diaphragmatic aspect.

In the text the lobes are designated cephalic, ventral, and caudal. In the right lung the cephalic lobe is relatively very large and has a separate bronchus. In Text-fig. 2 the small median or azygous lobe is shown. The normal lung tissue is unshaded; the heavily shaded circles represent necrotic tissue; the areas cross-hatched designate an older stage than the areas having parallel lines only.

Cultures were prepared from the different lobes on several media as described under Case 440. The extensive necrosis of lung tissue naturally led to the inference that a variety of bacteria would be found. This, however, was not the case. In none of nine horse serum tubes did any liquefaction suggesting *B. pyogenes* occur. In all but one, which remained sterile, *B. actinoides* multiplied in pure culture. Similarly the plain agar tubes containing bits of tissue and blood agar tubes developed pure cultures of *B. actinoides* with two exceptions in which a mold and a fleshy colony appeared.



The following case was the last of this particular outbreak.

No. 508.—Female calf, born May 7. Attendant gives a history of digestive disturbance (scours) beginning a few days after birth and lasting 3 weeks. When first seen June 3, the calf was emaciated, weighing 75 pounds. The respirations were rapid and shallow. Both nostrils were soiled with whitish, mucoid masses. The right ear was held low and was discharging a viscid, pus-like matter. The temperature fluctuated between 39° and 40°C. until June 7, when it was killed.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIGS. 3 and 4. Dorsal and ventral aspects of lungs of Calf 508. For explanation of the shading see Text-figs. 1 and 2.

The autopsy showed the usual lung involvement (Text-figs. 3 and 4). Three types of lesions are present: (1) the focal necroses; (2) a firm hepatization with slight enlargement of the cephalic and ventral lobes, which are furthermore permeated with grayish foci 1 to 2 mm. in diameter; and (3) a more recent stage, characterized by a uniformly dark red airless condition. The affected lobes are furthermore variegated by air-containing territories. The lesion referable to the right ear was not traced, owing to the injury inflicted by the blow necessary to stun the animal. A small cavity was found in the temporal bone filled with a puriform liquid which may have been associated with the lesion. Films from this fluid contained numerous polynuclears and several kinds of bacteria (diplococci, bipolar and minute bacilli). Cultures from various regions of the diseased lungs were made. In all a variety of bacteria developed, among them *B. pyogenes*. *B.*

*actinoides* was detected in all serum cultures. In two tubes the colonies of this bacillus were present in large numbers and among them scattering liquefying colonies of *B. pyogenes*. Pure cultures of *B. actinoides* could not, however, be obtained from these tubes. The sections of material fixed in Zenker's fluid did not show any details differing from those already described.

As an illustration of the condition of the lungs of an animal surviving this type of pneumonia, the following case from the first outbreak is of interest.

No. 6.—This calf was nearly 7 months old when killed. It was born in June, 1916. When received the calf was normal as to temperature, respiration, and pulse. It was thin and hide-bound, but not unusually weak. No early history was obtained. It was killed because unthrifty. The disease was restricted to the right cephalic lobe of the lung. It was adherent to surrounding structures by easily broken fibers. One-half of the lobe is permeated with yellowish white sacs containing a thick creamy fluid. The other (caudal) half of the same lobe presents a grayish mottling of regular pattern. The air tubes of the entire lobe are surrounded by broad, whitish bands, or zones, and contain viscid, glairy molds. The trachea contains a large amount of a viscid, flour-paste-like whitish matter. In sections of the affected lobe the air tubes stand out prominently as thickened tubes. The thickening is due to a broad envelope of plasma cells and new connective tissue around them, while what is left of the parenchyma is slightly emphysematous and contains scattering desquamated alveolar cells (Fig. 13). A number of partly occluding ingrowths into small air tubes are made up largely of an endothelial cell type with an admixture of polynuclears. In a mediastinal lymph node, associated with the diseased lobe, the normal lymphoid cells are almost entirely replaced by the plasma cell type. Cultures from the various lobes developed a rich growth of a bacterium which agreed in cultural characters with the bipolar type of organisms (*B. boviseplicus*). Its virulence towards rabbits was very low. At this time *B. actinoides* was not yet known to the writer. The lesions were ascribed to *B. boviseplicus*.

After an interval of 6 months following the second (1919) outbreak a sporadic case of pneumonia appeared, which is of importance in several directions.

No. 544.—Female calf, born Oct. 6, 1920. It was reported sick by attendant Nov. 18 and transferred to the Institute next day. The animal was emaciated and very weak, unable to stand. The respirations were rapid and shallow. The buttocks were soiled with feces. Temperature on Nov. 19 was between 41° and 41.8°C. during the day. The animal refused all food. Early Nov. 20 the attendant found the calf panting and grunting. It died soon after and was autopsied at 10 a.m.

Besides the extensive pneumonic changes, the only noteworthy lesion is a septic condition of the stumps of both iliac arteries. The lumina are patent and the intima is coated with a thin, grayish, pultaceous layer not removable by washing. One artery contains a grayish yellow, cylindrical thrombus about 2 cm. long. The lungs are extensively hepatized. Even the left caudal lobe is nearly one-half solidified. The cephalic and ventral lobes of both sides have dimensions about twice those of the normal collapsed lung. The hepatization is smooth, grayish red, excepting where the cut bronchi exude whitish, pasty masses. The hepatization of the azygous and the caudal lobes is of a dark red color, less firm. The pleura is free, adhesions absent. Necroses are not detected in any lobe. Both main bronchi contain flakes of thick, glairy matter filling the mouths of many branches. The trachea contains many roundish masses of the same exudate, pea size and coated with froth. Tubes inoculated from various affected lobes, about twenty in all, develop, with one exception, into pure cultures of *B. actinoides*.

The nature of the pathological process was not made clear in the sections examined. The invasion of the epithelium of the ultimate bronchioles shown so clearly in No. 440 could not be seen. The air tubes were plugged with cell masses extending into them from the alveolar ducts. The cells consisted largely of polynuclears, feebly tinted necrotic cells, and cells resembling polynuclears in staining but with roundish nuclei. Throughout the affected lung tissue cells of endothelial and plasma type were abundant and intermingled with the others. Owing to the congested condition of the capillary network their relation to the alveolar walls was not clear. Cloud-like masses of minute bacilli were present among the cell masses and resolved with difficulty into their elements.

To bring this case into relation with those already described it may be assumed that the infecting agent was introduced and disseminated through the lungs in such large numbers and deposited in so many places that the process did not have time to reach the stage of focal necrosis before the calf died.

To the information gained by the two groups of cases in the same large herd may be added some data obtained from the autopsies of calves used for producing small-pox vaccine in the vaccine laboratory of the Massachusetts State Board of Health in Boston. During the year 1908 a small number of pneumonic lungs were found in calves killed after the vaccine had been removed. They weighed between 150 and 200 pounds.

*No. A.*—Both cephalic lobes are pneumonic. The affected tissue is flesh-red with lighter mottling or else simply collapsed. The air tubes contain molds of creamy pus. Sections show the stage of general polynuclear infiltration of parenchyma and the filling up of minute air tubes with the same kind of cell masses.

In one section there is a cellular irruption into an air tube and in the proliferated mass are colonies of minute bacilli.

*No. B.*—Both cephalic and ventral lobes are consolidated, and there is also a large focus in the left caudal lobe. Involved tissue is grayish red to grayish yellow, delicately and regularly mottled. The air tubes are distended with whitish, viscid plugs. The process in the focus in the caudal lobe is freshest, that in the free tips of the ventral lobes oldest. In general the histological picture is that of collapse, and infiltration with polynuclears. In one section there are two contiguous foci of cell exudation and hemorrhage undergoing necrosis. Many colonies of fine bacilli are in the periphery of these foci (Fig. 10). These cases, as far as the information goes, suggest an underlying process like that initiated by *B. actinoides*.

#### *The Pathogenic Action of Bacillus actinoides.*

The bacteriological study of spontaneous cases pointed to *Bacillus actinoides* as the primary inciting agent of the bronchopneumonia with *Bacillus pyogenes* and more rarely, in older calves, *Bacillus bovisepiticus* settling down in the occluded air tubes and necrotic lung tissue. The next step was to determine whether cultures of this bacillus, under suitable conditions, could produce lesions like those found in spontaneous cases. In the following pages is given a brief statement of observations made on inoculated calves, since small mammals had thus far shown themselves quite insusceptible. Some of the calves were inoculated subcutaneously, some intravenously, others into the trachea. The area of the operation was shaved, cleansed with water and alcohol, and painted with tincture of iodine. In the intratracheal injections, a small incision was made through the skin over the trachea before inserting the needle.

*No. 467.*—Bull calf, born Jan. 21, 1920. Dam had placenta retained at birth and swab from uterus as well as agglutination test was positive for *B. abortus*. Temperature, taken twice daily, fluctuated between 38.5° and 39°C. until Feb. 3, the day of inoculation.

The turbid condensation water of a horse serum culture of *B. actinoides* from Calf 462, 5 days old and under artificial cultivation since Jan. 7, was drawn off, making about 1 cc. in all. This was increased to 3 cc. by the addition of sterile bouillon. The fluid was injected into the subcutis in front of right shoulder. The temperature rose to 39.8°C. within 3 hours and remained at the same level next day. On this day a local swelling appeared over the point of inoculation about 1½ inches in diameter and ¼ to ⅓ inch thick. The temperature fell below 39°C. on Feb. 8. The swelling increased slightly.

On Feb. 19, a second inoculation was made, this time into a jugular vein with the same strain used for the first, subcutaneous inoculation. The condensation water was ground slightly in a crucible to break up the flocculi and calf serum water was added to dilute the suspension. The temperature rose to 40°C. within 3 hours and gradually fell to normal during the night. No further elevation occurred. The calf was killed Mar. 4. The weight had risen from 88 to 125 pounds. The local swelling was about the size of a small hen's egg. The calf was stunned with a heavy blow, a clamp quickly placed on the trachea, and the neck vessels were severed. During this operation the local swelling ruptured and several cubic centimeters of a thick, whitish mass, like flour-paste, were discharged. The autopsy showed normal organs, except for two very small foci of collapse, one in the right cephalic, the other in a caudal lobe of the lungs. The local swelling is adherent to the overlying skin, but readily dissected from the subjacent fascia. The under surface of the capsule is sprinkled with minute hemorrhages. It is 3 to 4 mm. thick, pearly white on section and enclosing a cavity partly filled with a soft, odorless mass like flour-paste. The inner surface of the capsule is dark red to hemorrhagic and delicately mammillated. The cavity has small recesses or pockets. The puriform contents are readily removed from the entire wall. The associated prescapular lymph node is about one-fourth larger than the opposite node and on section smooth and quite juicy. The contents of the abscess consist of cells, only a few of which retain the stain. These are mononuclear. Scrapings from the capsule show a mixture of polynuclear and mononuclear elements and occasional cloud-like masses of minute bacilli. Some of the mononuclear cells contain groups of minute bacilli. *B. actinoides* was recovered from the abscess both on horse serum and on agar plus blood. In one of the latter the growth appeared richer than usual and was found by inoculation into two guinea pigs and by microscopic examination to contain *B. abortus*. The presence of *B. abortus* is accounted for by the infection of the dam which had been demonstrated by suitable tests after the birth of the calf.

*No. 336b.*—Bull calf, born Feb. 28, 1920. On Mar. 9, it weighed 111½ pounds. A horse serum culture from Calf 462, 5 days old, was used. The condensation water, which contained a dense suspension of flocculi visible to the naked eye and shown to be pure by the microscope, was removed to a sterile tube, 5 cc. of bouillon were added, and the whole was thoroughly shaken. The flocculi were still visible after the shaking. The suspension was injected into a jugular vein at 4 p.m. The temperature rose from 38.8° to 40.6°C. in 5 hours and gradually dropped to 38.9°C. during the night. The calf was kept under observation for 36 days. During this period the temperature remained between 38° and 39°C. and the calf's general condition was normal. On the 36th day it weighed 145 pounds and was sold for slaughter.

*No. 479b.*—Bull calf, born Feb. 26. Inoculated on Mar. 9, when 12 days old. A first culture from the local lesion of Calf 467 on agar plus blood, 5 days old, was used. The surface growth was transferred with a platinum loop to 5 cc. of bouillon until a fine suspension was produced. This was made up of excessively fine

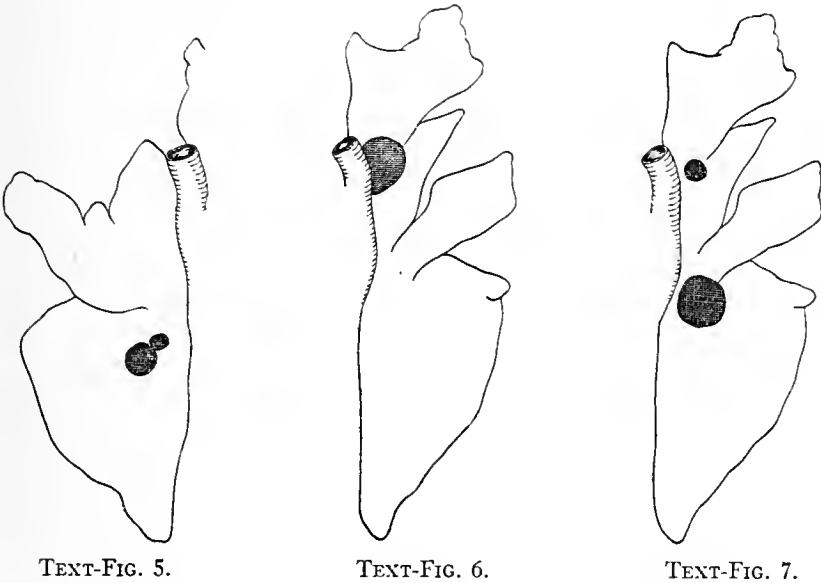
granules when viewed in a strong light. It was injected into a jugular vein. Calf weighed at this time 97½ pounds. The temperature rose from 38.7° to 40°C. within 5 hours. It was only a few tenths above normal next day. The calf continued normal in condition. The temperature remained normal.

On Mar. 24, 15 days after the intravenous injection, a second inoculation was made, this time into the trachea. The condensation water of three horse serum cultures of *B. actinoides*, 4 days old, from Calf 467 was removed to a sterile tube and enough bouillon added to make 12 cc. in all. 5 cc. of this were injected. Within 24 hours a swelling appeared over the trachea, of undefined borders, probably 6 inches long and 2 inches broad. After several days this swelling was somewhat reduced and was now found to be made up of two indurations, one over the point of injection, the other lower down at root of neck. Apr. 5. The right leg was shown to have restricted motion and to be painful when handled. Apr. 15. The right shoulder appeared a trifle larger than the left and tender. The lameness increased. The calf, showing no general symptoms, was killed Apr. 20, 27 days after the intratracheal injection. The only lesions found were the local swellings. One about 2 inches in diameter is situated just caudad of the larynx; a second, 4 inches in diameter, just caudad of the first. Both are firm, tense, slightly fluctuating. They are covered by muscles of the neck and attached to the cervical portion of the thymus. Both are sacs with walls 5 to 10 mm. thick, smooth interiorly, and filled with a soft odorless mass, resembling cottage cheese. They communicate with each other through a narrow opening. The contents consist of lymphocytes and polynuclears, the former greatly predominating. Both kinds of cells largely refuse the stain. There are also filamentous masses, probably necrotic fibers. In the larger abscess a strip of necrotic tissue was found in the caseous contents about 4 inches long, representing the remnants of a vessel about 2 mm. internal diameter. After washing away the pus the external surface was found covered with small papilla-like projections.

On the prominence of the right shoulder joint there is a swelling about the size of an egg, easily dented by pressure. The tumor is situated between the tendinous ends of the muscles inserted in the scapula. There is marked thickening of the intermuscular fascia radiating from the capsule of the tumor to a depth of several inches. On incision of the thick capsule, an irregular cavity is exposed, with contents mucoid, whitish, quite unlike those of the tumors over the trachea. However, films show the same cell elements, the difference being due to the advanced necrosis of the contents of the other abscesses.

Sections of the abscess walls show a mass of muscular and connective tissue with marked hyperplasia of the connective tissue and some fibrin. There is also a general infiltration with cells of endothelial character. The pus attached to the inner wall consists wholly of cells of endothelial type. Some of these are filled with minute bacilli. There are also clouds of free bacilli feebly stained and easily overlooked. Cultures prepared from both abscesses over the trachea develop into pure growths of *B. actinoides*. No cultures were made from the abscess on the shoulder. The respiratory tract as well as the remaining viscera was normal.

No. 495b.—Female calf, born Apr. 15, 1920. On May 11, 10.30 a.m., when 26 days old and 88 pounds in weight, it was inoculated into the trachea. The condensation water of six horse serum cultures from Calf 479b, 5 days old, was drawn into a fresh tube and 6 cc. of bouillon were added, making 13 cc. of heavily clouded fluid. 8 cc. of this were injected. Animal coughed occasionally after



Effect of the intratracheal injection of cultures of *B. actinoides*.

TEXT-FIG. 5. Necrotic focus in left caudal lobe of Calf 520.

TEXT-FIG. 6. Similar focus in right cephalic lobe of Calf 474b.

TEXT-FIG. 7. Two necrotic foci, one in cephalic, the other near root of right ventral lobe of Calf 495b.

the operation. The temperature rose about 1°C. during the day and was down again the next day. 3 days later the temperature reached 40°C. and fluctuated between 40.2° and 40.7°C. for 6 days, then dropped to 38.3° to 38.7°C. The calf weighed 99 pounds on May 27, having gained 11 pounds in 16 days. It was killed on this day.

The digestive tract is normal throughout. In the respiratory tract there is a bit of semitranslucent mucus on one vocal cord. In the lower trachea there is a similar mass, pea size, made up of alveolar epithelium and polynuclear leucocytes. There are two groups of focal necroses in the lungs (Text-fig. 7). On the dorsal ridge of the right caudal lobe, near root of the right ventral, there

is a small, whitish, opaque, subpleural mass. On palpation, other firm nodules are felt embedded, about half a dozen in all. On section the contents show as thick, cheesy cores in dense capsules. The tissue between them is still air-containing. There is no fresh pneumonia or collapse around them. A similar cheesy nodule is in the right cephalic lobe, embedded in emphysematous tissue. Besides these two necrotic foci there are several collapsed lobules in different lobes. Lymph nodes of thorax are normal. There are 10 cc. of clear fluid in pericardial sac. Both kidneys are spotted everywhere with whitish areas, discrete and confluent, occupying fully one-half of the total cortex. These spots are the bases of cones of fan-shaped outline on section, extending to the medulla. The substance of these foci is glistening, smooth, almost like cartilage in appearance. Urine taken from the bladder soon after death is very pale, clear, faintly alkaline, specific gravity 1.004. Boiling after adding 1 to 2 drops of acetic acid yields a very faint cloud. The liver is slightly fatty.

Sections of the necrotic foci in the lungs show centrally a nucleus of dead lung tissue in which the alveolar structure is still preserved (Figs. 15 and 16). These nuclei are 1.5 to 2 mm. in diameter. In the central core the alveoli are nearly empty. In the peripheral zone, they are filled with a fine granular material in which many cells are embedded. Immediately surrounding the necrotic center is a broad zone in which the lung structure is occasionally seen, but is chiefly replaced by cellular debris embedded in a granular matrix. Dense groups of minute bacilli are in the outer layers of this zone where many alveolar cells are still recognizable. Outside this zone is a layer of fibroblasts and newly formed capillaries. The whole is enclosed in a mantle of lymphoid cells, occupying the meshes of the compressed lung tissue. In the vicinity of the necrotic focus there are zones of lymphocytes around the ultimate and next larger bronchioles, associated with collapse and broadening of alveolar walls through cell infiltration. In some air tubes small groups of polynuclears are seen. Sections of the kidney lesions show interstitial hyperplasia with shrinkage and disappearance of glomerular tufts, dilatation of some convoluted tubules and disappearance of others. Cultures made from the necrotic lung foci both on horse serum and blood agar developed the characteristic appearances of *B. actinoides*. Only one contained also a fine filamentous growth, probably a streptothrix. All of sixteen cultures prepared from kidney tissue remained free from growth.

No. 474b.—Black and white bull calf, born Apr. 21, 1920. June 3. The calf weighed 135 pounds. The condensation water of three horse serum cultures, 4 days old, showing a dense crop of flocculi was withdrawn into a sterile tube, about 4 cc. in all. This was injected into the trachea, and without removing the needle, about 6 cc. of sterile salt solution were injected through it. After the calf had been placed on its feet it coughed some, ejecting a fine spray. Following the injection the temperature rose about 1.5°C. It was normal next day. The calf was killed June 21, 18 days after the inoculation. The autopsy showed normal conditions with the following exceptions. In the abdominal cavity there are



about 50 cc. of a clear, slightly yellowish fluid which coagulates into a jelly-like mass on standing. Between the vocal cords, along the trachea, and at the root of the right supernumerary bronchus are small masses of a semi-opaque, mucoid substance, consisting of polynuclears, alveolar, and endothelial elements embedded in mucus. The lungs show quite generally over all lobes scattering, very small, dark reddish collapsed or pneumonic foci from mere points to 1 mm. in diameter and of various shapes. In the right cephalic lobe near root is a consolidation about 3 cm. in diameter, but more or less squarish and extending through depth of lung tissue (Text-fig. 6, page 457). It feels lumpy. On section the lumps appear as spherical, firm, grayish foci, about 1 cm. in diameter and centrally necrotic. There is congested lung tissue between them. Each focus is made up of a very firm, almost cartilaginous capsule. Within is a viscid, very thick, whitish mass, which in the larger foci contains in it a more grayish, spongy nucleus found to be necrotic lung tissue. The layer around this contains many mononuclear elements whose cytoplasm, in many cells at least, is filled with fine rods. Masses of similar rods are free in the stained film. The necrotic masses are odorless. Cultures made from the necrotic foci on horse serum showed the characteristic flocculi in 3 days. All contained in addition molds with delicate mycelium. Pure subcultures of *B. actinoides* were obtained, however, from surface colonies. Cultures on blood agar were also prepared. Some were pure growths of *B. actinoides*, others contained one or several other types of colonies. At the same time, cultures were prepared from the air-containing, normal lung tissue by introducing into culture tubes small bits of lung tissue. Molds, liquefying colonies, and several other types of colonies appeared in the tubes.

*No. 504b.*—Black and white female calf, born June 10, 1920. Weight, June 21, 110 pounds. On June 28, the condensation water of six serum tubes of *B. actinoides* from Calf 474b, 3 days old, was brought together in a sterile tube. The heavily clouded fluid, 5 cc. in all, containing flocculi was injected into the trachea at 3 p.m. The temperature rose about 1.5°C. within 4 hours, then slowly subsided. No abnormal conditions appeared, and it was killed 23 days after inoculation. At this time it weighed 136½ pounds. No lesions were found in the respiratory tract or elsewhere.

*No. 520.*—Guernsey calf, male. Born Oct. 8, 1920. Weight on Oct. 22, 83½ pounds. On Oct. 27, four horse serum tubes inoculated from cultures of Calf 474b, 4 days ago, were used for intratracheal injection. The condensation water was drawn up into a sterile tube, a little salt solution being used to wash it out more thoroughly. 5 cc. of a moderately clouded fluid were collected in this way. This was injected into the trachea at 10.30 a.m. Before removing the needle, 10 cc. of sterile Ringer's solution were injected. The temperature rose from an average of 38.6° to 41°C. 7½ hours after the injection and gradually fell during the night—the temperature being taken every 2 hours—to 38.8°C. next morning. Following the injection the calf began to appear depressed, the respirations were slightly irregular, about 90 at 2.30 p.m. Attendant noticed a slight chill between 4.30 and 5.30 p.m. The temperature did not go above normal again and the

calf, showing no signs of any disturbance thereafter, was killed Nov. 12. The weight on this day was 96 pounds. The organs were normal with the following exceptions.

Beginning about 5 cm. below lower margin of the larynx, the mucosa covering six intercartilaginous spaces of the trachea is dark red over each space. There are no signs of swelling. In the left caudal lobe a firm mass is buried within air-containing, slightly emphysematous lung tissue (Text-fig. 5, page 457). It is about 1 cm. in diameter. Soft, smooth, flour-paste-like material oozes out from a slight incision. The mass is placed in Zenker's fluid. A film of this thick mass shows necrotic cells and no bacteria. There is a second 2 to 3 mm., firm, yellowish white focus near the first. It contains a nucleus of necrotic lung tissue, enveloped in the same material found in the larger focus. Films of this contain large numbers of minute bacilli among cells of endothelial and leucocyte type which hold the stain as if still living. Cultures were not prepared from this case, owing to the scanty material and the need for histological examination of the lesions. This did not, however, show more than has been given from the other cases in which intratracheal injection of *B. actinoides* produced focal necrosis. The central nucleus of the necrotic tissue was surrounded by a layer of polynuclear leucocytes and outside this a connective tissue capsule was forming. Between these two layers, within a narrow zone, colonies of minute bacilli were abundant. Many groups of bacilli were within the cytoplasm of cells.

The results of the inoculation of cultures of *Bacillus actinoides* may be briefly summarized. The subcutaneous injection leads promptly to a large swelling which becomes very firm. After several weeks the mass of the swelling becomes necrotic, the contents caseous, and a thick, firm wall forms with hyperplasia of the connective tissue in the immediate environment of the focus. Softening of the capsule, ulceration of the overlying skin, and discharge outward take place within 4 weeks. In several cases, in which the injections were into the trachea, a small amount of the culture fluid entering, accidentally, the tissues overlying the trachea led to large abscesses. The swelling is primarily due to an increase of mononuclear, endothelial-like elements, with later invasion of polynuclears. The final product is a soft mass, like cottage cheese in appearance, consisting of small lumps, the whole embedded in a thick mass, smooth, glistening, like flour-paste in consistency. In one abscess the remnant of a vessel, 4 inches long, was embedded in the mass. Clouds of minute bacilli are found in the zone outside the necrotic mass where the cellular elements are still stainable. In one case (No. 479b) a second (meta-static) abscess developed at some distance from the site of inoculation.

Injection into the circulation in one case failed to produce any lesions. The second case was sold in excellent condition, probably without lesions. Injection into the trachea failed in two out of five cases to produce any appreciable changes in the lungs. In the remainder there developed small necrotic foci, single or multiple, identical both macroscopically and microscopically with those occurring in spontaneous cases.

The distribution of the induced lesions did not agree entirely with that of spontaneous cases, thus meeting in part the possible objection that the lesions might have been due to naturally acquired infection. The difference in localization was probably due to the fact that the calves were lying on their backs when the culture fluid was injected. This abnormal position may have led to a different drainage of the injected fluid from that occurring in spontaneous cases.

In one instance (No. 495b) the lung lesions were associated with multiple focal scleroses of the kidney cortex.

The culture used was obtained from Calf 462 and passed in succession through Nos. 467, 479b, 495b, 474b, and 520. This was done with the expectation that the virulence would maintain itself. From every one of the above, excepting No. 520, pure cultures were recovered. None were made from the last case.

It will be noted that the intratracheal inoculations produced what was called above the first stage of the disease only. The lesions were restricted in extent and the secondary lesions due to dissemination of the bacilli from the primary necrotic foci did not take place. Perhaps some special depressing conditions may be needed to continue the disease into the clinical stage.

In contrast to the very acute, both destructive and tissue-stimulating action of *Bacillus actinoides* in the subcutis of calves is the following negative action on one sheep.

*No. 164.*—Barren ewe. On May 11, 1920, the same suspension of cultures from Calf 479b used on No. 495b was injected under the skin in front of left shoulder. Neither local nor thermic reaction followed. The experiment was closed June 1.

*Correlation of the Pathological and Bacteriological Data.*

The foregoing observations and experiments demonstrate the existence of a specific bronchopneumonia causing mortality in calves chiefly in the 2nd and 3rd months of life. In animals which die within the 1st month other agencies due to fetal conditions and to infections acquired during or soon after birth may involve the lungs. Surviving cases affected with a chronic pathological condition of one or more lobes, characterized by purulent bronchial exudation, abscess formation, and fibrosis may be met in the 4th to the 6th month or even later. As a rule, the disease invades both lungs symmetrically. The parts affected first are the smaller cephalic and ventral lobes, more particularly the dependent portions (Text-figs. 1 to 4). With the progress of the disease the involvement moves upwards towards the dorsal border of these lobes and backwards into the azygous lobe and the caudal lobes. Death takes place when one-half of the latter have become airless. The pleura is involved only where necroses extend to the surface. Here adhesions to surrounding structures form from the capsules of the resulting abscesses.

Several kinds of lesions are presented in the ordinary acute case. The distal two-thirds of the cephalic and ventral lobes are, as a rule, considerably enlarged beyond the normal collapsed state, very firm, dark or light reddish in color. Regularly sprinkled in this ground are grayish, 1 to 2 mm. areas. The proximal third of the same lobes and the affected regions of the caudal lobes are but little enlarged, uniformly dark red, less firm, and without the grayish mottling. Careful search, by manipulation if necessary, reveals scattering firm masses, sometimes deeply embedded, more commonly extending to one or both lung surfaces. They vary from 2 to 10 mm. in diameter. Several may coalesce. They consist of a pearly, dense capsule containing necrotic lung tissue, enveloped in a layer of viscid pus. These sequestra are with rare exceptions located in the cephalic and ventral lobes. They are probably among the oldest lesions. They and the mottled pneumonic regions which are always associated with them are either developed at the same time or else the necroses are the source of the infection which produces the pneumonic condition. The smooth dark red pneumonia is secondary to the other lesions,

probably through the agency of aspirated purulent exudates. The distribution and extent of the three conditions are shown in the text-figures. The pneumonic condition is usually not universal in the affected lobes. Small or large air-containing territories may occur in them, chiefly along the free margins of the lobes. The one universal characteristic lesion is the filling of the air tubes of the affected lobes with a thick, viscid, glairy, white, mucopurulent matter.

The microscopic characters of the different stages are fairly well definable. The formation of the necroses (Figs. 12, 15, and 16) has not been traced, owing to lack of material in the early stages. Associated with or following these, there is a filling up of the alveoli with several cell types to form the mottled pneumonic territories. At the same time the ultimate bronchioles and alveolar ducts may become involved. The entire parenchyma becomes filled with mononuclear elements, probably a mixture of alveolar cells, endothelial and lymphoid cells. The polynuclear leucocyte is absent in this stage. The smooth, fresher pneumonic condition which develops later is due to partial or complete filling up of alveoli and bronchioles with polynuclear leucocytes. In these the epithelium remains intact. With this stage there appears the zone of plasma cells around the air tubes filled with cell debris (Figs. 7 and 13). Numerous polynuclear leucocytes are found moving outward through the epithelium into the lumina. This accumulation of plasma cells is probably due to the cell debris in the lumina of the air tubes. They are present after a certain time whether one or several species of bacteria are at work. That they are the result of the stimulus exerted by toxins and other products absorbed from the disintegrating cellular plugs in the lumina seems to be at present the most plausible explanation of their presence. The minute grayish dots permeating the older pneumonic lesions, which are so striking in the fresh lung of most but not all cases, have not been associated definitely with any microscopic details. They may represent the earlier proliferative lesions in the ultimate bronchioles in some cases and the gathering of plasma cells in later stages.

Before summarizing the results of the bacteriological studies it should be stated what is apt to be forgotten in an interpretation of results, that the normal lungs of calves, and other species as well,

contain a considerable variety of living microorganisms. When bits of normal lung tissue are placed in culture tubes, growth appears quite invariably. Spore-bearing bacilli, various kinds of molds, and streptothrix are among the commonest forms detected. In the isolation of *Bacillus actinoides*, on agar, bits of lung tissue are essential to growth. On coagulated serum tissue is not necessary. It is somewhat surprising that so many cultures obtained from bits of diseased lung tissue were pure cultures of *Bacillus actinoides*. It would seem as if under the influence of the violent tissue reaction the banal forms found in normal lungs are largely destroyed.

The bacteriological examination has shown a variety of results, depending on the stage of the pneumonic process, the rapidity of its development, and whether the animal died or was killed. In the 1919 to 1920 epidemic, twelve cases were investigated. *Bacillus actinoides* was the only cultivable organism present in five of these. It was present but associated with other pathogenic species in four more. It was not detected in the remaining three. *Bacillus pyogenes* was present in seven cases but not in pure culture. *Bacillus bovisepiticus* was present in three or four cases, always with a variety of other species. Of the five cases in which *Bacillus actinoides* was exclusively present, two had died and three were killed. Of the four in which it was associated with other species, three were killed and one died. Of the three in which it was missed, two died and one was killed. A study of the brief protocols suggests that *Bacillus actinoides* was missed through overgrowth with other species in certain very acute dead cases and in the more chronic surviving cases.

In the tissues of the diseased lungs *Bacillus actinoides* is found in the peripheral layer of lung sequestra, in colonies in the cell masses occupying alveoli and alveolar ducts (Figs. 8 to 11), and in the proliferated epithelium obstructing the bronchioles less than 0.1 mm. in diameter (Figs. 1 to 7). This latter process which is rather unique and which has not thus far been described in the pathology of pneumonias was especially well brought out in sections of No. 440, but it could be detected in most other cases after more or less searching. In the invaded epithelium *Bacillus actinoides* appeared with the sheath or capsule around it. In other situations it was usually free from capsules and appeared in dense, cloud-like colonies. It should

be stated here that, with rare exceptions, air tubes over 0.1 mm. in diameter had their epithelium intact. The cell masses with which many were filled—the glairy, mucopurulent contents—consisted of cell debris moving up from the ultimate bronchioles and the parenchyma.

After injection of pure cultures into the trachea *Bacillus actinoides* has thus far produced only the early stage of necrosis (Text-figs. 5 to 7 and Figs. 15 and 16). The diffuse secondary and later lesions characterizing the spontaneous fatal and very sick cases killed were absent. The relation of *Bacillus actinoides* to the primary necrotic lesions is thus placed beyond doubt. That it is also responsible for the general pneumonic involvement is not proved by experiment but made highly probable. Neither *Bacillus pyogenes* nor *Bacillus bovisepiticus* is responsible, since these easily cultivated bacteria were absent in five advanced cases. Considerations based on bacteriological and histological studies are sufficient to throw them out of the other cases in which they were found, except in the rôle of continuing a disease fully under way by multiplying in the necrotic tissues and the bronchial cell debris. If *Bacillus pyogenes* and *Bacillus bovisepiticus* are not responsible for the diffuse pneumonic lesions, then either *Bacillus actinoides* or else some non-cultivable, unrecognized microorganism is. The writer has throughout assumed that *Bacillus actinoides* is the sole responsible agent.

A study of the mode of response or reaction of the tissues to *Bacillus actinoides* has not come within the scope of the present investigation. The finding of some small susceptible species is necessary to provide material for such study. In general the reaction in the lungs is at first associated with mononuclear cell types, either endothelial or lymphoid or both. In later stages polynuclear cells completely dominate the process. This change may be due to an immunity reaction. Many things point to this explanation. Thus the later smooth pneumonic condition is always associated with the polynuclear cell type. In those animals in which the process halts with the necroses, the remaining parenchyma contains only polynuclears in focal distribution. Moreover, *Bacillus actinoides* is only very rarely detected with the microscope in the lesions associated with polynuclear cells, although cultures reveal its presence. This rarity suggests that the active stage of multiplication is over.

The various factors entering into the process such as quantity and dissemination of the original infecting material, relative susceptibility of the host, and the rapidity with which immune forces are called forth determine the extent and rapidity of the early, usually necrotic changes and the promptness of dissemination of the virus from such necrotic foci over new territories. Added to these factors are species of bacteria, chiefly *Bacillus pyogenes* and *Bacillus bovisepiticus*, which may graft themselves on the diseased tissues. In thus restricting *Bacillus bovisepiticus* to a secondary position, the author does not imply that there may not be virulent races of *Bacillus bovisepiticus* capable of initiating outbreaks of pneumonia.

Aside from Case 6, no detailed description of the cases occurring in the 1917 outbreak, which was the basis of an earlier publication,<sup>1</sup> has been given. This group of cases had not been worked up with so much care and in such detail partly because the very first case which came to autopsy yielded pure cultures of *Bacillus bovisepiticus* and the developing epizootic was considered due to this bacillus. With the succession of cases, the appearance of a polymorphic bacillus and its association with a closely resembling species, *Bacillus pyogenes*, rendered orientation very difficult until a pure culture of *Bacillus actinoides* was made to grow indefinitely in subcultures and its forms clearly differentiated from *Bacillus pyogenes*. A repeated study of the accumulated data has shown such a parallelism between the 1917 and the 1919 cases in gross and minute anatomy and histology of the affected lungs and in the bacteriology that any review of these early cases would be in the main a repetition of what precedes. The underlying etiological factor and the processes it initiates are the same in both groups of cases.

A study of the epidemiology of the pneumonia due to *Bacillus actinoides* needs much additional material. The source of this micro-organism is not yet defined. It will, however, be safe to regard partly recovered older animals of the same species as the purveyors of the virus. The termination of certain lesions in necrosis followed by copious discharge from the resulting abscesses opens the way for the virus outward. It is probable that unrecognized cases occur in which the process does not go beyond localized necrosis of small territories. Such cases may serve in maintaining the mild disease



until the cold season favors the secondary extension of the lesions and brings acute clinical cases to the surface. This view is supported by the production of necroses experimentally in calves without any appreciable rise in temperature or other suspicious deviations from health.

Owing to the meager descriptions given in reports of outbreaks of pneumonia in calves, it has been impossible to identify this form of bronchopneumonia in earlier writings. An attempt was made in the article on *Bacillus actinoides*<sup>1</sup> to correlate the latter with Lignières' actino-bacillus which produces subcutaneous and other abscesses but not pneumonia. A continued study of *Bacillus actinoides*, however, does not make the relationship seem any more real. It remains to study calf pneumonias in both anatomical and microbiological directions afresh, since the existence of several etiological types of lung disease is probable. The anatomical and histological study of these pneumonias is important, since information gained in this way tends to restrain hasty conclusions concerning the etiology where several different species of bacteria may find opportunity for multiplying in the lungs in the course of the disease.

#### CONCLUSIONS.

A bronchopneumonia of calves in the early months of life is described and its etiology associated definitely with a minute bacillus, *Bacillus actinoides*. *Bacillus pyogenes*, *Bacillus bovisepiticus*, and, less frequently, staphylococci and streptococci may appear later in the affected lungs.

Subcutaneous injections of cultures of *Bacillus actinoides* produce large indurations ending in necrosis. Similarly intratracheal injections produce circumscribed necroses of lung tissue. The cultivation of *Bacillus actinoides* and its morphological peculiarities have been sufficiently described and illustrated in an earlier publication<sup>1</sup> to ensure success on the part of those who attempt to isolate it.

The writer is indebted to Dr. R. B. Little for assistance in bringing together the clinical data.

## EXPLANATION OF PLATES.

## PLATE 42.

FIG. 1. Section of lung tissue, Calf 440, showing dilatation of a small bronchus and complete filling up of the lumen with the characteristic exudate. The epithelium has disappeared from most of the circumference. The exudate is intimately associated with the subepithelial tissues. The parenchyma around the bronchus is partly collapsed, partly pneumonic. The capillaries are distended with corpuscles, and the alveoli are filled with cells of endothelial and lymphoid type. See Fig. 4 for magnification of a part of this exudate.  $\times 240$ .

## PLATE 43.

Both figures from Calf 440.

FIG. 2. An air tube about 0.1 mm. in diameter with ingrowth of cellular masses, which are filled with bacilli, as shown in Fig. 5. The section passes through a dividing bronchus, of which one branch is involved. Remnants of the epithelial cells are traceable in the irrupted mass which contains cells with round, pycnotic nuclei.  $\times 240$ .

FIG. 3. Transection of an air tube about 0.3 mm. in diameter. The exudate is intimately connected with the subepithelial tissue of the bronchus in two places. Elsewhere the epithelium is still *in situ*. The exudate is of the same character as in Figs. 1 and 2. It is permeated with cloud-like masses of minute bacilli showing only as slightly darker patches in the photograph.  $\times 310$ .

## PLATE 44.

FIG. 4. A portion of the diseased bronchus of Fig. 1, enlarged  $\times 1,000$ . The bacillar and capsulated or club-shaped forms are present, filling the cytoplasm of the cell mass which has nearly plugged the lumen of the small bronchus.

FIG. 5. A portion of the diseased bronchus of Fig. 2, enlarged  $\times 1,000$ . The bacillar, cloud-like masses have filled the cytoplasm of the cells as in the preceding figure. The club-shaped forms are shown in the lower right-hand region in many cross-sections and rather sparsely elsewhere in the bacterial masses.

## PLATE 45.

FIG. 6. From Calf 440. Section through a small bronchus, of which the epithelium along one margin (to the right in the figure) is still *in situ*. The other margin is obliterated by an ingrowth which nearly occludes the lumen. This ingrowth is densely permeated with *B. actinoides*. The bacilli appear chiefly as very short rods of irregular size and form throughout the section not taken up by the bronchial epithelium.  $\times 1,000$ .

FIG. 7. From the same lung. The small air tube is nearly occluded by the mass of cells on the left. This mass is permeated densely with *B. actinoides* in bacillar form. In addition, larger circular areas, suggesting capsulated cocci, are sprinkled through the mass. These are identified as club-shaped forms of *B. actinoides* cut across.  $\times 1,000$ .

## PLATE 46.

FIG. 8. Section through the parenchyma of the lungs, Calf 440. The alveolus is filled with cells. In the center of the cell mass is a colony, or floccule, of *B. actinoides*.  $\times 1,000$ .

FIG. 9. Two alveoli, each containing a floccule. The clubs are shown imperfectly in optical cross-section.  $\times 1,000$ .

## PLATE 47.

FIG. 10. Section from an old case, designated as Calf B on page 454 of the text showing two foci in which are cloud-like colonies of minute bacilli, appearing as irregular darker patches in the figure.  $\times 1,000$ .

FIG. 11. Section from the lungs of Calf 440. In the upper left-hand corner is a large cloud-like colony of bacilli. Other colonies in the field are out of focus and appear as slightly darker patches.  $\times 1,000$ .

## PLATE 48.

FIG. 12. Sequestrum from the lung of Calf 462, slightly dislodged. The outlines of the alveoli may still be distinguished in the upper portion of the necrotic mass. The border of the wall or capsule above, which suggests epithelium in the figure, is compressed tissue made up of fibroblasts and plasma cells.  $\times 25$ .

## PLATE 49.

FIG. 13. Section from the lungs of No. 6. The more or less deformed bronchi are filled with polynuclear leucocytes and surrounded by a broad zone of plasma cells and fibroblasts.  $\times 925$ .

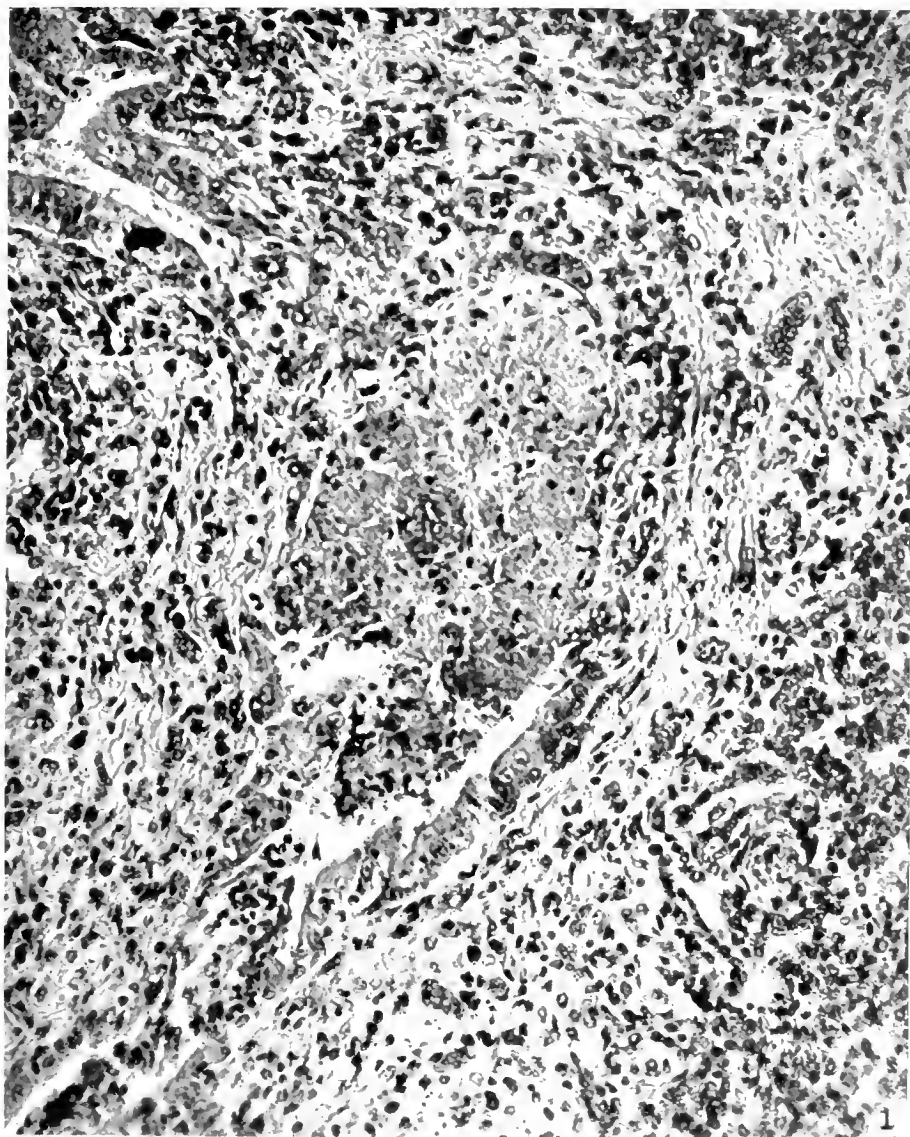
FIG. 14. Section of lung tissue from Calf 446. This case is somewhat older than No. 440. Near the center of the figure is a more or less deformed bronchiole containing polynuclear leucocytes. The lung tissue in a broad zone around this tube is occupied by groups and masses of plasma cells and polynuclear leucocytes.  $\times 925$ .

## PLATE 50.

FIG. 15. Represents a necrotic focus from the lungs of Calf 495b (see Text-fig. 7) which received an intratracheal injection of *B. actinoides*.  $\times 60$ .

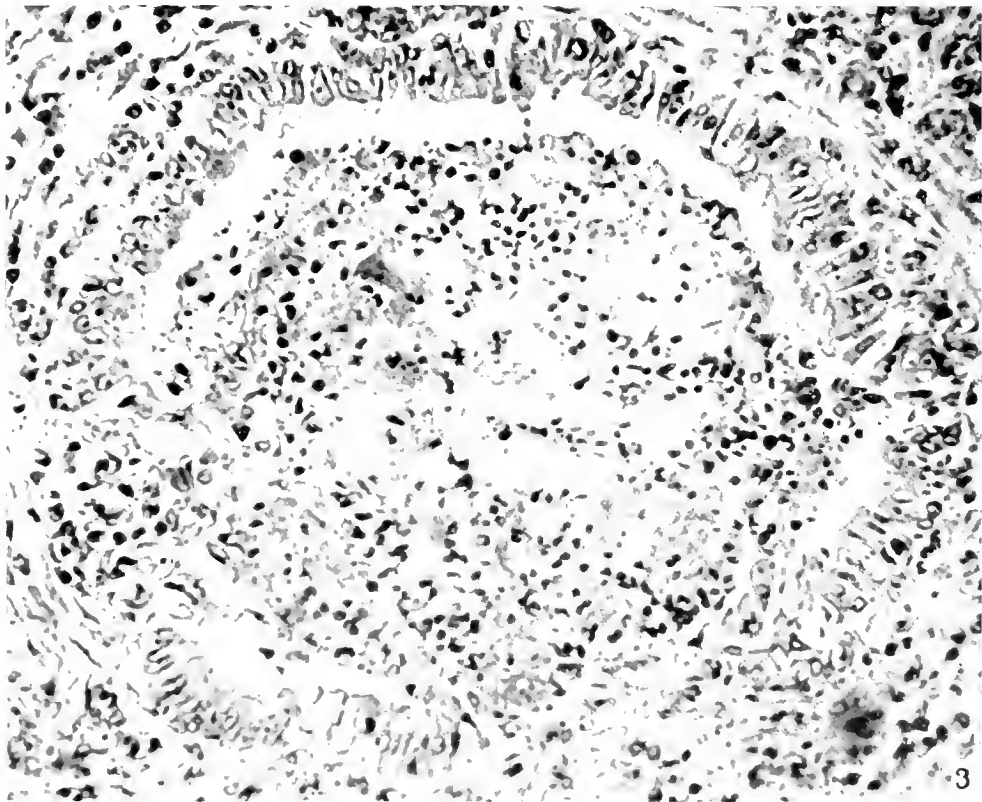
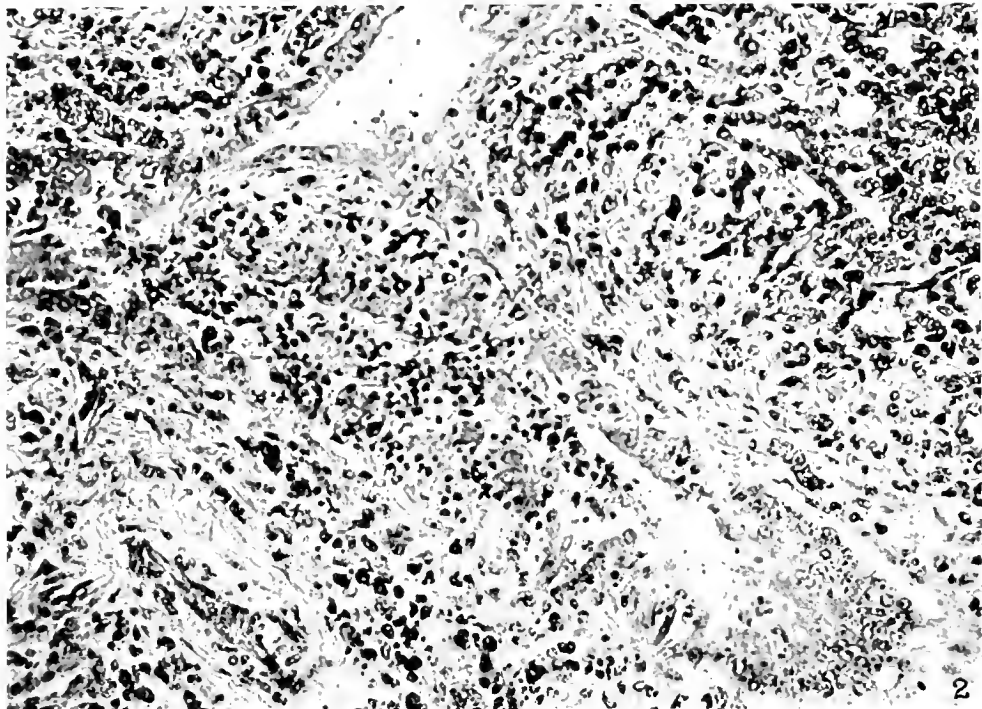
FIG. 16. Enlargement of the zone just outside the necrotic mass shown in Fig. 15. Irregular groups of *B. actinoides* occupy this zone. The large group on the right has only a few bacilli in focus, whereas the central group is fairly distinct.  $\times 1,000$ .





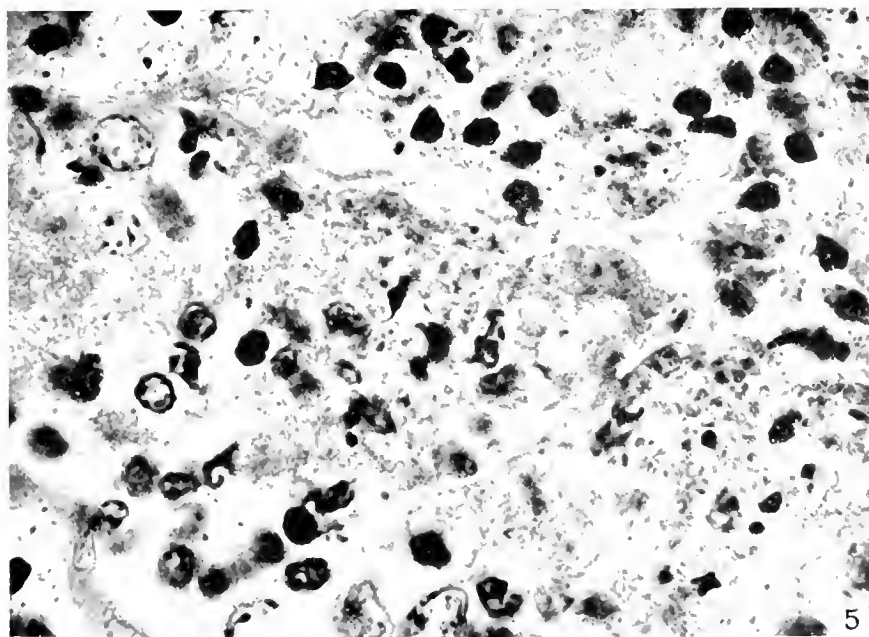
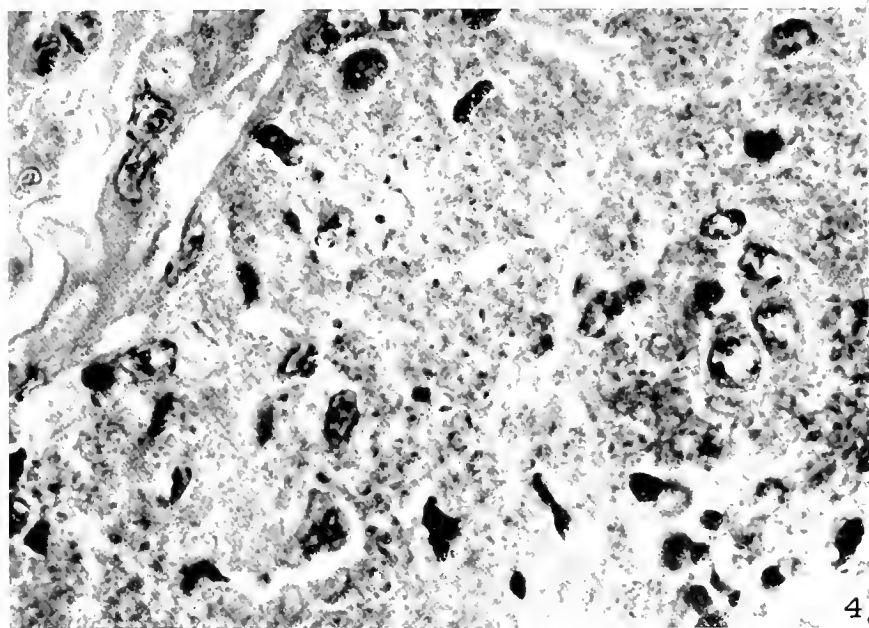
(Smith: *Buillus actinoides*.)





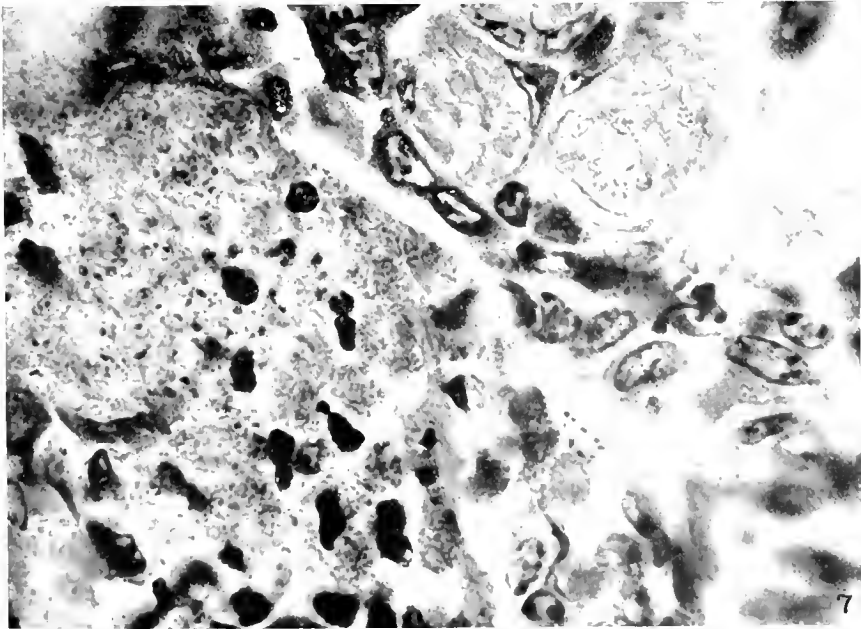
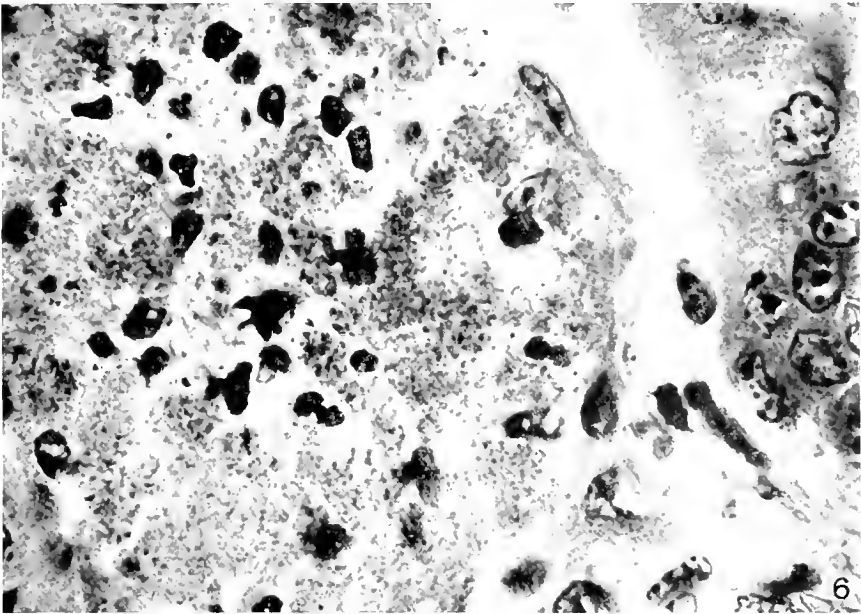






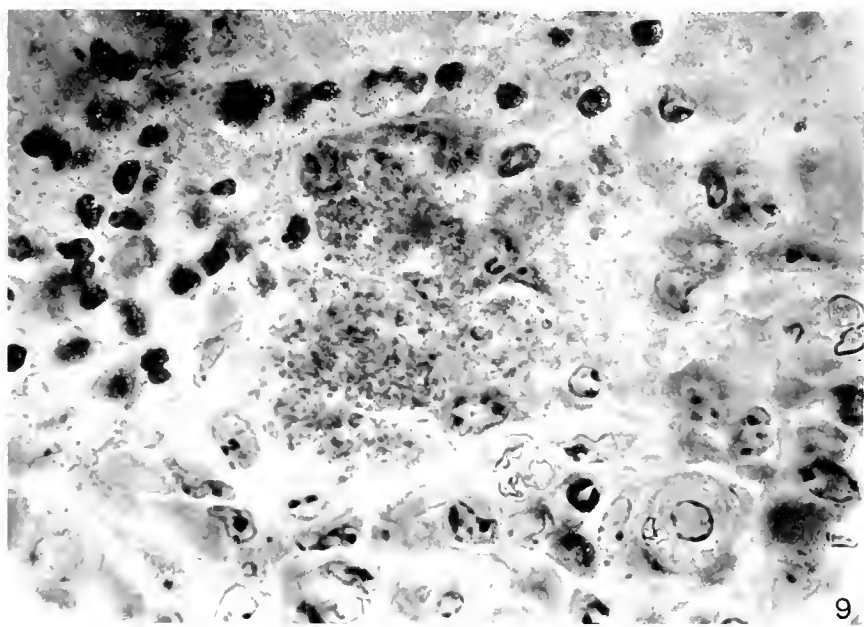
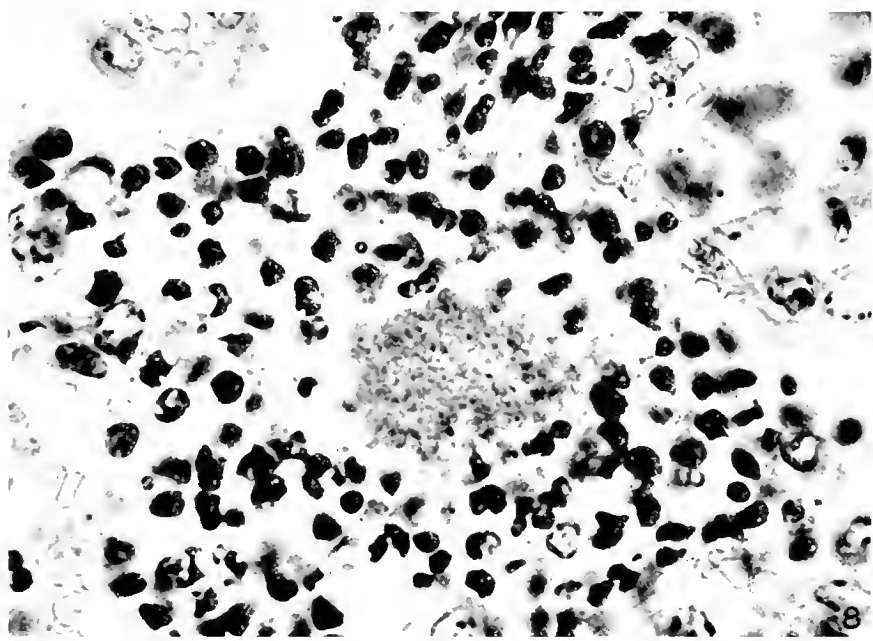
(Smith: *Bacillus actinoides*.)





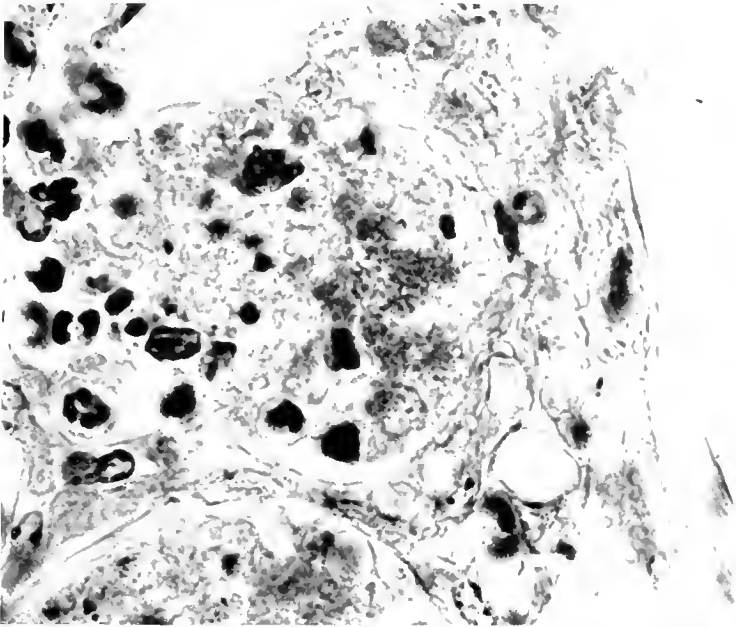
(Smith: *Bacillus actinoides*.)



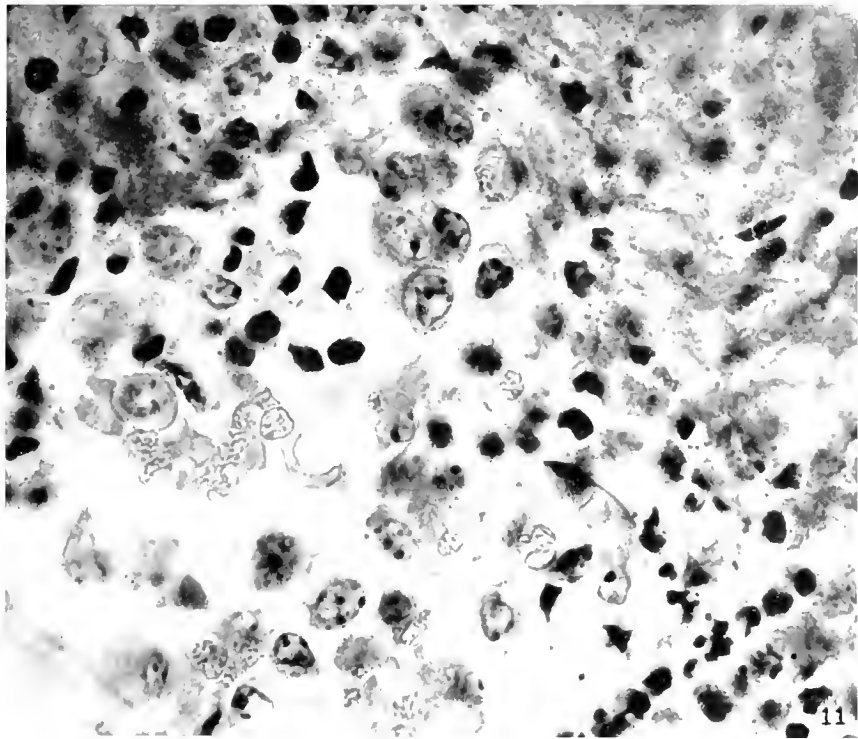


(Smith: *Bacillus actinoides*.)





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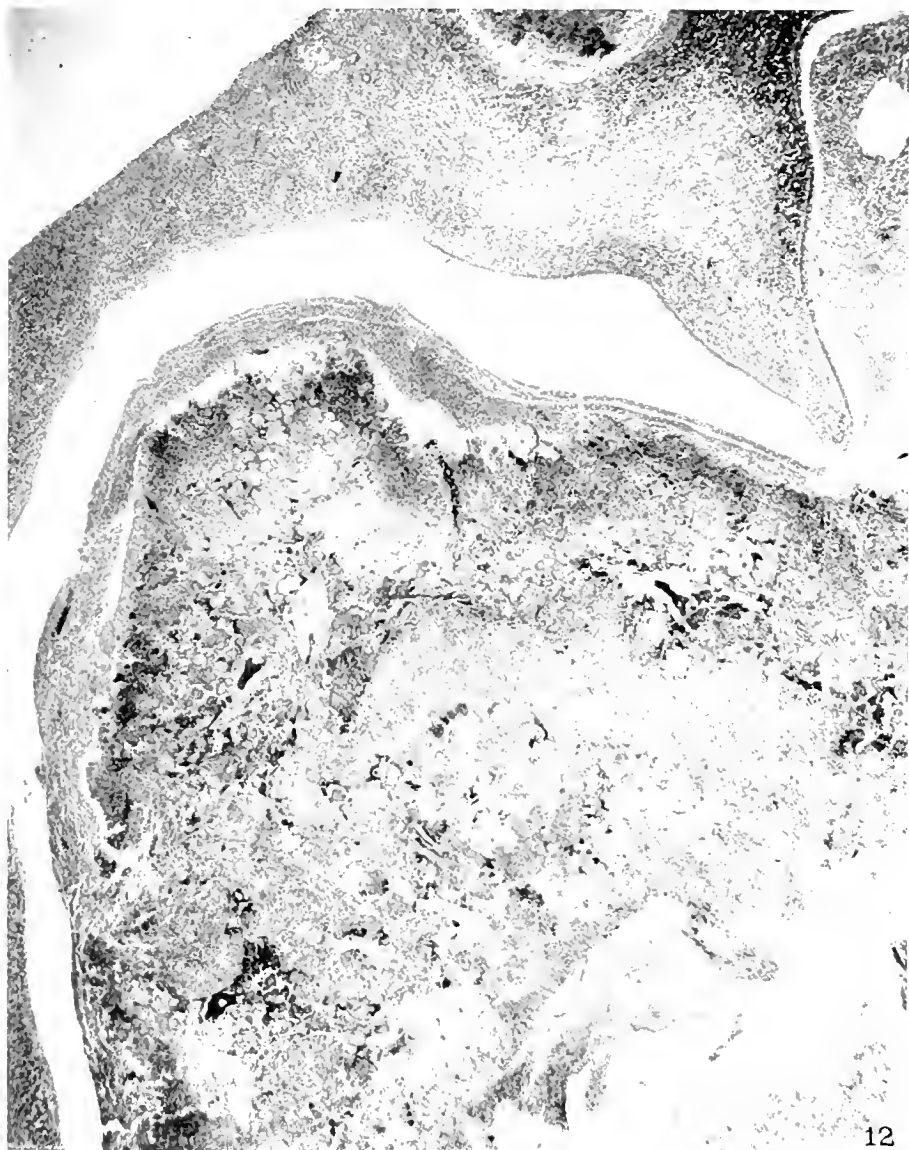


11

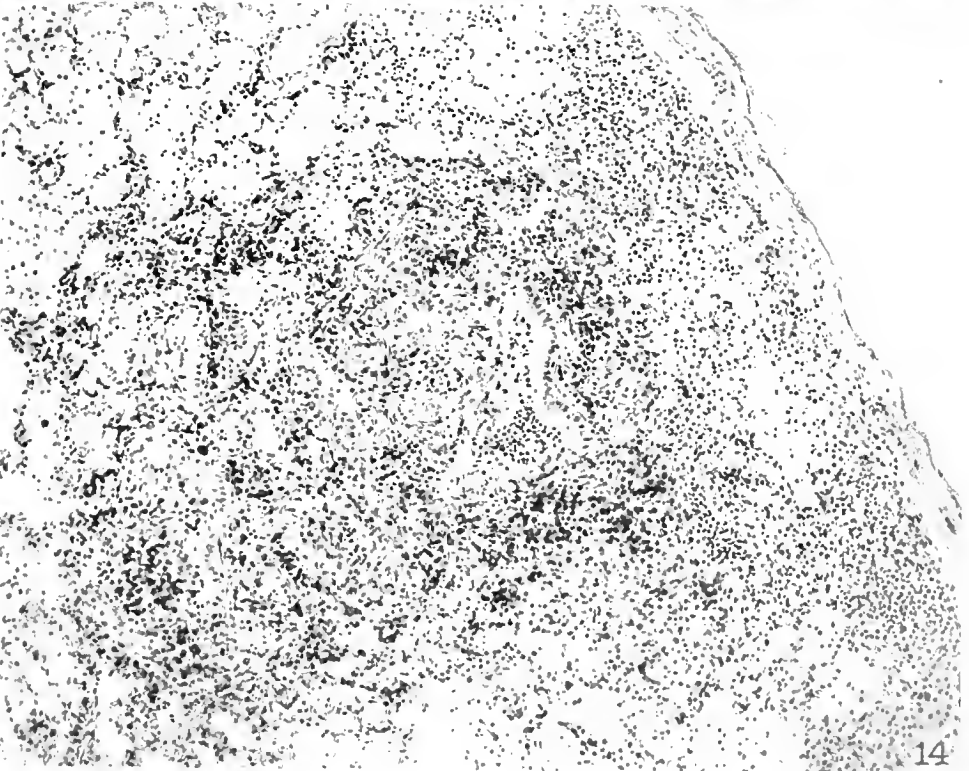
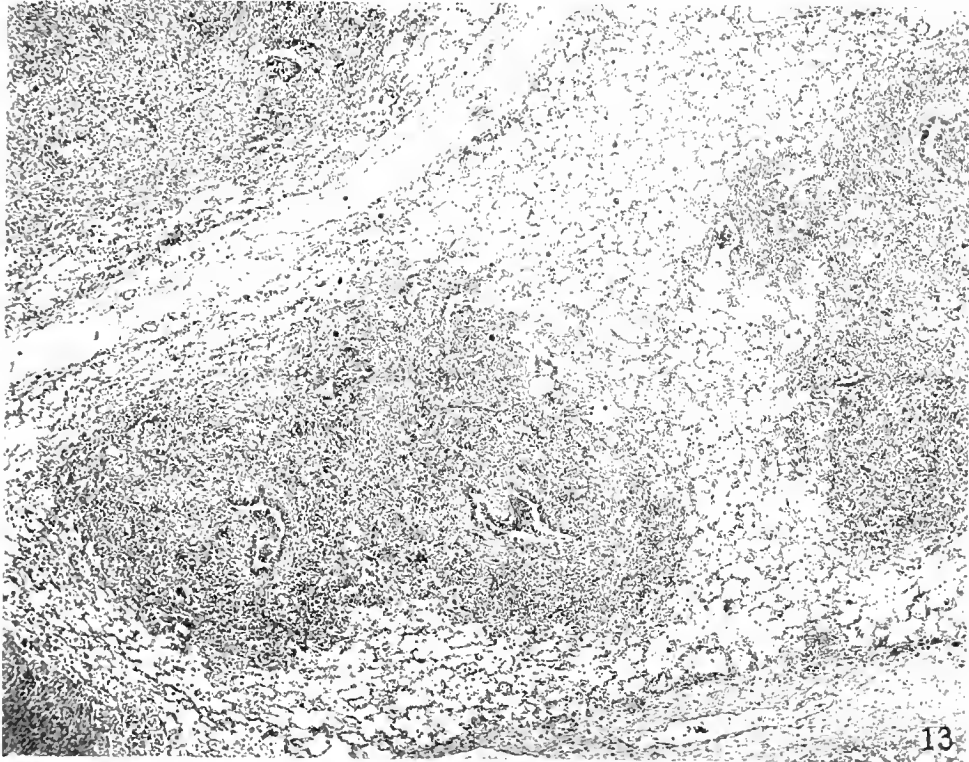
(Smith: *Bacillus actinoides*.)



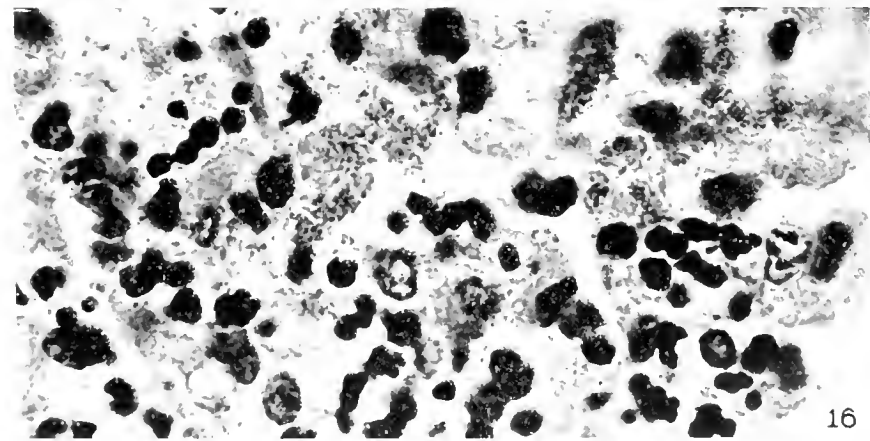
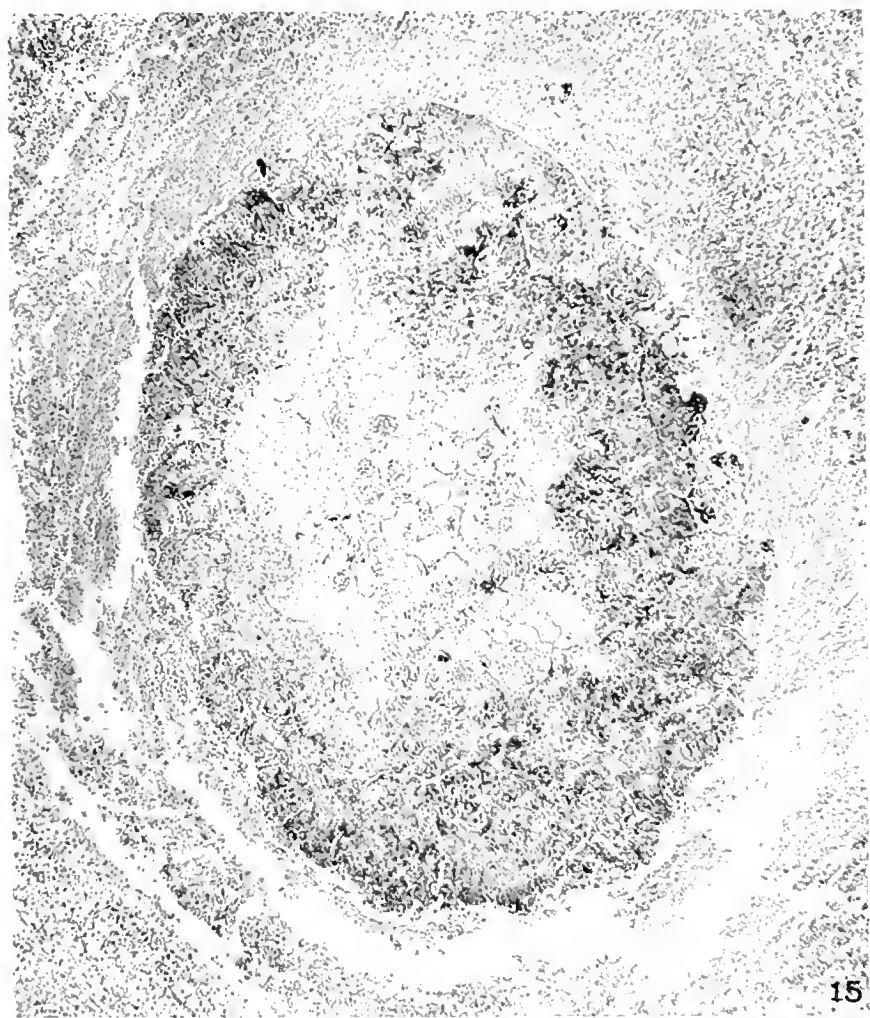














## PRECIPITIN RESPONSE IN THE BLOOD OF RABBITS FOLLOWING SUBARACHNOID INJECTIONS OF HORSE SERUM.

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(Received for publication, January 18, 1921.)

During the treatment of cases of cerebrospinal meningitis with antimeningococcic serum in a large army hospital<sup>1</sup> a peculiar reaction was repeatedly observed. This reaction appeared in patients who, after having received several intraspinal injections of serum, were given serum intravenously. While the injection was being made into the blood stream, or immediately afterward, some of the following signs and symptoms were frequently noted: flushing, sudden feeling of warmth, and restlessness, followed by pallor, dyspnea, cyanosis, vomiting, and prostration. Hypodermatic injections of epinephrine and atropin brought relief. In the press of work there was no opportunity for detailed blood pressure and temperature studies. These manifestations, apparently anaphylactic, occurred only when several intraspinal treatments were given before initial intravenous serum therapy, and had no relation to the time of the last intraspinal injection. They were not noted when combined intraspinal and intravenous therapy was applied from the outset. Similar observations are described by Stone and Truitt<sup>2</sup> in a report of a large series of cases of cerebrospinal meningitis, and Haden<sup>3</sup> mentions intolerance to antimeningococcic serum under like conditions.

With this experience in mind, horse serum was injected into rabbits intraspinally and the resulting precipitin formation in the blood

<sup>1</sup> Hospital of the American Embarkation Center, Le Mans, France.

<sup>2</sup> Stone, W. J., and Truitt, R. C. P., *Arch. Int. Med.*, 1919, xxiii, 282.

<sup>3</sup> Haden, R. L., *Arch. Int. Med.*, 1919, xxiv, 514.

was compared with that induced by similar intravenous injections. In a few instances anaphylactins were studied.

The formation of specific precipitins, in the blood, to foreign proteins was first recorded by Kraus<sup>4</sup> in 1897. Since then, numerous methods have been described for inducing the formation of these bodies in high titer.<sup>5, 6, 7</sup> Protein is injected into susceptible animals, either *via* the blood stream or peritoneum, the methods differing chiefly in dose and interval.

Weed<sup>8</sup> has clearly shown the pathway by which substances injected into the subarachnoid space readily find access to the blood stream. On the other hand, most substances, including circulating toxins and antibodies (agglutinins to *B. typhosus*, tetanus toxin, and hemolyticamboceptors)<sup>9, 10, 11</sup> are normally excluded from the spinal fluid by means of the meningeal-choroid complex which acts as an effective barrier to them. Artificial damage to the meninges, however, by such agents as normal horse serum and even normal salt solution, makes them permeable to immune bodies. Flexner and Amoss<sup>12</sup> thus recovered neutralizing principles to the virus of anterior poliomyelitis from the spinal fluid, after intravenous injection into monkeys. More recently, Amoss and Eberson<sup>13</sup> by similar methods detected meningococcic agglutinins in the cerebrospinal fluid after their introduction into the blood stream.

#### EXPERIMENTAL.

Normal horse serum, without preservative, was used throughout the experiments. It was injected into the subarachnoid space of rabbits by introducing a No. 24 Luer needle attached to a glass syringe, through a sterile field just below the occipital ridge in the midline. The needle was carried forward and slightly downward until it punctured the occipito-atlantoid ligament. A yield of from 0.5 to 1 cc. of cerebrospinal fluid was thus readily obtained. The needle was left in place, the syringe disconnected, and a second syringe

<sup>4</sup> Kraus, R., *Wien. klin. Woch.*, 1897, x, 736.

<sup>5</sup> Kolmer, J. A., *A practical text-book of infection, immunity and specific therapy*, Philadelphia and London, 2nd edition, 1917.

<sup>6</sup> Zinsser, H., *Infection and resistance*, New York, 2nd edition, 1918.

<sup>7</sup> Hektoen, L., *J. Infect. Dis.*, 1917, xxi, 279.

<sup>8</sup> Weed, L. H., *Anat. Rec.*, 1917, xii, 461.

<sup>9</sup> Mott, F. W., *Lancet*, 1910, ii, 1.

<sup>10</sup> Ransom, F., *Z. physiol. Chem.*, 1900, xxxi, 282.

<sup>11</sup> Cushing, H., *J. Med. Research*, 1914-15, xxxi, 1.

<sup>12</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499.

<sup>13</sup> Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1919, xxix, 597.



with the correct amount of serum was attached. After slightly withdrawing the plunger to prevent any chance puncture of a vessel the serum was slowly injected.

Precipitin tests were made by mixing 0.3 cc. of rabbit serum with 0.3 cc. of normal horse serum (for unchanged horse serum, equal parts of an anti-horse rabbit serum with dilutions of the serum or cerebrospinal fluid to be tested). After incubation at 37°C. for 1 hour in a water bath the tubes were placed in the ice chest over night and readings were made the following morning. As controls, normal rabbit serum and normal sheep serum were used. In all instances as controls for rabbits receiving subarachnoid injections other rabbits were injected with identical amounts of the same serum intravenously, and the bleedings and precipitin tests of each were done at the same time.

The following protocols are selected as typical of the results obtained. Text-figs. 1 to 3 are graphic charts of precipitin tests in each experiment.

*Experiment 1.*—Rabbit 1; weight 1,860 gm. Feb. 6, 1920. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. No reaction.

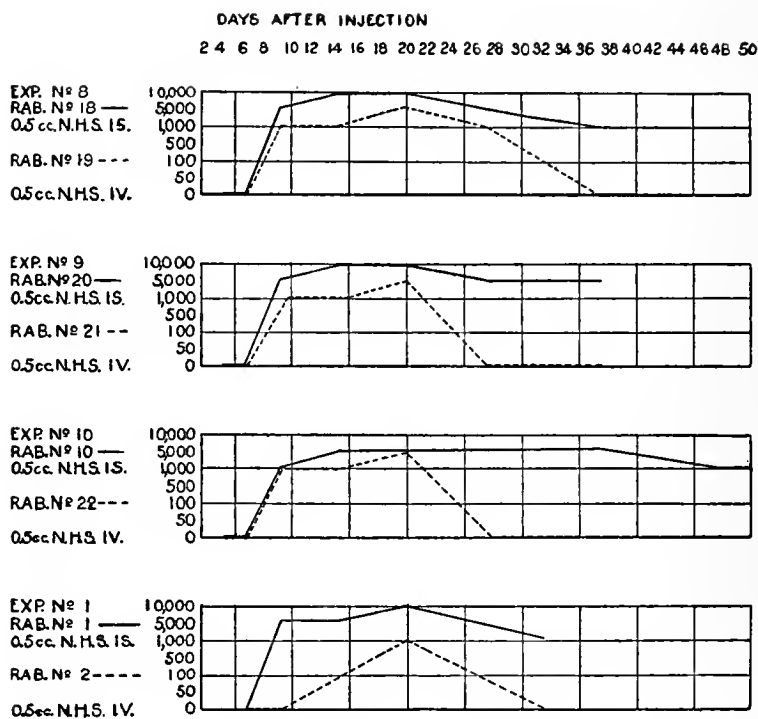
Rabbit 2; weight 1,650 gm. Feb. 6, 1920. 0.5 cc. of normal horse serum injected intravenously.

*Precipitin Tests with Sera 1 and 2 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 1.	Serum 2.	Serum 1.	Serum 2.	Serum 1.	Serum 2.	Serum 1.	Serum 2.	Serum 1.	Serum 2.	Serum 1.	Serum 2.
1920												
Feb. 12.....	0	0	0	0	0	0	0	0	0	0	0	0
“ 15.....	≠	≠	+++	≠	++	0	++	0	+	0	≠	0
“ 20.....	0	+	+	+	++	≠	++	0	+	0	0	0
“ 26.....	++	0	++	+	++	++	++	+	++	≠	+	0
Mar. 9.....	≠	0	+	0	++	0	++	0	—	0	0	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

In the tables +++ indicates very heavy precipitate; ++, heavy precipitate; +, distinct precipitate; ≠, questionable precipitate; 0, no precipitate; —, no test.



In the text-figures N.H.S. indicates normal horse serum; I S., intraspinally; I V., intravenously.

TEXT-FIG. 1. Precipitin tests after one intraspinal or intravenous injection of normal horse serum.

*Experiment 2.*—Rabbit 3; weight 2,100 gm. Mar. 30, 1920. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. No reaction.

Rabbit 4; weight 2,100 gm. Mar. 30, 1920. 0.5 cc. of normal horse serum injected intravenously.

*Precipitin Tests with Sera 3 and 4 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 3.	Serum 4.	Serum 3.	Serum 4.	Serum 3.	Serum 4.	Serum 3.	Serum 4.	Serum 3.	Serum 4.	Serum 3.	Serum 4.
<i>1920</i>												
Apr. 5.....	0	0	0	0	0	0	0	0	0	0	0	0
" 8.....	≠	0	++	+	++	+	++	0	+	0	+	0
" 13.....	+	0	++	+	++	+	++	+	+	+	+	0
" 19.....	0	0	+	+	++	++	++	+	+	+	+	0
" 26.....	+	0	+	0	+	0	+	0	≠	0	0	0
May 3.....	0	0	+	0	+	0	≠	0	0	0	0	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

*Experiment 3.*—Rabbit 5; weight 1,920 gm. Mar. 30, 1920. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. No reaction.

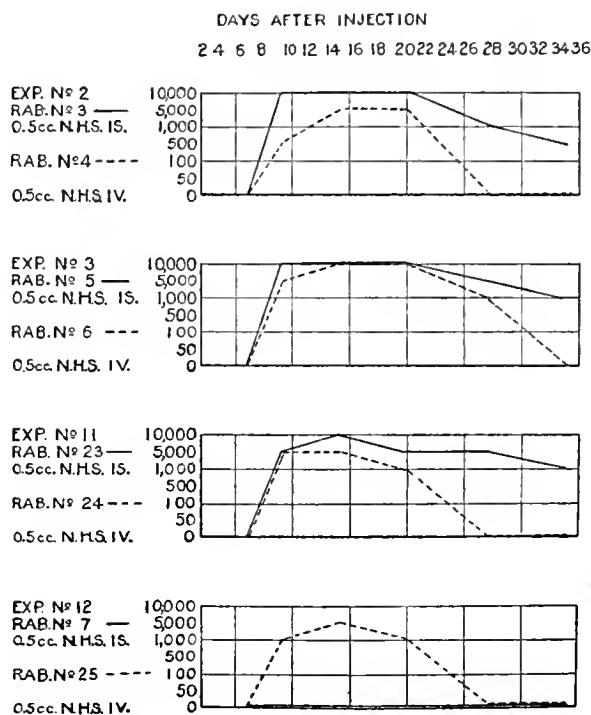
Rabbit 6; weight 2,000 gm. Mar. 30, 1920. 0.5 cc. of normal horse serum injected intravenously.

*Precipitin Tests with Sera 5 and 6 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 5.	Serum 6.	Serum 5.	Serum 6.	Serum 5.	Serum 6.	Serum 5.	Serum 6.	Serum 5.	Serum 6.	Serum 5.	Serum 6.
<i>1920</i>												
Apr. 5.....	0	0	≠	0	+	0	≠	0	0	0	0	0
" 8.....	++	+	++	+	+++	+	++	+	++	+	+	≠
" 13.....	+	0	++	++	++	++	++	++	++	+	+	+
" 19.....	0	≠	++	++	++	++	++	++	++	+	+	+
" 26.....	≠	0	++	+	++	+	++	+	+	≠	0	0
May 3.....	++	0	++	0	++	0	+	0	≠	0	0	0

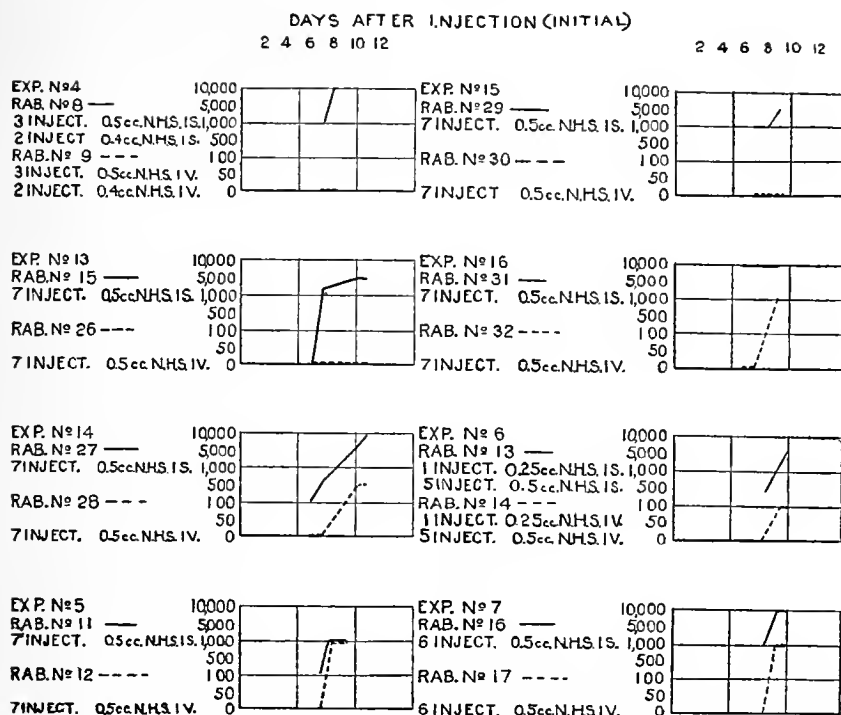
Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

In these experiments, in which single injections of normal horse serum were made intraspinally and intravenously, precipitins appeared in the blood stream in both instances on approximately the same days. Rabbits receiving serum intraspinally, however, showed precipitins in higher titers, which persisted after the disappearance of precipitins in rabbits treated intravenously. In the one instance



TEXT-FIG. 2. Precipitin tests after one intraspinal or intravenous injection of normal horse serum.

that was followed precipitins after intraspinal injections were present in the blood in a titer of 1:1,000 on the 56th day after injection, whereas in the control rabbit, inoculated intravenously, they had disappeared by the 27th day. The single exception appeared in Rabbit 7, in which no precipitins were found after subarachnoid injections, whereas the control showed a normal precipitin curve



TEXT-FIG. 3. Precipitin tests after repeated intraspinal or intravenous injections of normal horse serum.

(Text-fig. 2). It is common to find rabbits which fail to give any precipitin response whatever to protein injections.<sup>5</sup>

In the next series of experiments repeated intraspinal injections of normal horse serum were given to rabbits; as controls others were so treated intravenously. The following protocols are typical.

*Experiment 4.*—Rabbit 8; weight 2,500 gm. Jan. 14, 1920. Subarachnoid puncture. 0.7 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. Jan. 15. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. Jan. 17. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.5 cc. of normal horse serum injected. Jan. 20. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid removed. 0.4 cc. of normal horse serum injected. Jan. 22. Subarachnoid puncture. 0.2 cc. of cerebrospinal fluid removed. 0.4 cc. of normal horse serum injected.

Rabbit 9; weight 2,350 gm. Jan. 14, 1920. 0.5 cc. of normal horse serum injected intravenously. Jan. 15. 0.5 cc. of normal horse serum injected intravenously. Jan. 17. 0.5 cc. of normal horse serum injected intravenously. Jan. 20. 0.4 cc. of normal horse serum injected intravenously. Jan. 22. 0.4 cc. of normal horse serum injected intravenously.

*Precipitin Tests with Sera 8 and 9 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.
1920												
Jan. 21.....	+	0	+++	0	++	0	+	0	≠	0	0	0
" 22.....	+++	0	+++	0	-	0	+++	0	++	0	+	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

*Precipitin Tests with Dilutions of Sera 8 and 9 against Serum 10, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Blood.*

Date.	1: 10		1: 100		1: 500		1: 1,000	
	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.	Serum 8.	Serum 9.
1920								
Jan. 15.....	+	-	+	-	0	-	-	-
" 17.....	++	-	+	-	≠	-	0	-
" 21.....	≠	++	0	+	0	0	0	-

*Precipitin Tests with Dilutions of Cerebrospinal Fluid 8 against Serum 10, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Cerebrospinal Fluid.*

Date.	1: 10	1: 100	1: 500	1: 1,000
1920				
Jan. 15.....	++	+	0	-
" 17.....	++	+	+	0

*Experiment 5.*—Rabbit 11; weight 1,780 gm. Mar. 2, 3, 5, 6, 8, 9, and 10, 1920. On each of these days a subarachnoid puncture was made, 0.5 cc. of cerebrospinal fluid was withdrawn, and 0.5 cc. of normal horse serum injected.

Rabbit 12; weight 1,900 gm. Mar. 2, 3, 5, 6, 8, 9, and 10, 1920. On each of these days 0.5 cc. of normal horse serum was injected intravenously.

*Precipitin Tests with Sera 11 and 12 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.
1920												
Mar. 9.....	≠	0	+	0	≠	0	0	0	0	0	0	0
" 10.....	+	0	++	+	++	+	+	+	0	0	0	0
" 11.....	++	+	++	++	++	+	+	+	0	0	0	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

*Precipitin Tests with Dilutions of Sera 11 and 12 against Serum 1, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Blood.*

Date.	1: 10		1: 100		1: 500		1: 1,000	
	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.	Serum 11.	Serum 12.
1920								
Mar. 8.....	≠	+++	0	+	0	0	0	0
" 9.....	+	+++	≠	++	0	≠	0	0
" 10.....	+	++	0	0	0	0	0	0

*Precipitin Tests with Dilutions of Cerebrospinal Fluid 11 against Serum 1, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Cerebrospinal Fluid.*

Date.	1: 10	1: 100	1: 500	1: 1,000
1920				
Mar. 8.....	0	0	0	0
" 9.....	++	+	≠	0
" 10.....	+	+	0	—

*Experiment 6.*—Rabbit 13; weight 1,960 gm. June 15, 1920. Subarachnoid puncture. 0.5 cc. of cerebrospinal fluid was removed. 0.25 cc. of normal horse serum injected. June 17, 18, 19, 21, and 23. On each of these days a subarachnoid puncture was made, 0.5 cc. of cerebrospinal fluid was removed, and 0.5 cc. of normal horse serum injected.

Rabbit 14; weight 2,100 gm. June 15, 1920. 0.25 cc. of normal horse serum was injected intravenously. June 17, 18, 19, 21, and 23. On each of these days 0.5 cc. of normal horse serum was injected intravenously.

*Precipitin Tests with Sera 13 and 14 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.
1920												
June 23. ....	±	0	+	0	+	0	0	0	0	0	0	0
" 24. ....	+	±	++	+	++	0	+	0	0	0	0	0
" 25. ....	++	+	++	+	++	±	+	0	+	0	0	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

*Precipitin Tests with Dilutions of Sera 13 and 14 against Serum 15, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Blood.*

Date.	1: 10		1: 100		1: 500		1: 1,000	
	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.	Serum 13.	Serum 14.
1920								
June 21. ....	+	+	0	+	0	0	0	0
" 22. ....	+	++	0	+	0	0	0	0
" 23. ....	±	++	0	+	0	0	—	0

*Precipitin Tests with Dilutions of Cerebrospinal Fluid 13 against Serum 15, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Cerebrospinal Fluid.*

Date.	1: 10	1: 100	1: 500	1: 1,000
1920				
June 21. ....	+	0	0	0
" 22. ....	+	0	0	0
" 23. ....	±	0	0	—



June 25, 1920, 2.30 p.m. Guinea Pig 1, 1 cc. of Rabbit Serum 13<sup>14</sup> intraperitoneally. Guinea Pig 2, 2.5 cc. of Rabbit Serum 13 intraperitoneally. Guinea Pig 3, 1 cc. of Rabbit Serum 14<sup>14</sup> intraperitoneally. Guinea Pig 4, 2.5 cc. of Rabbit Serum 14 intraperitoneally. June 26, 4 p.m. Guinea Pig 1, 0.5 cc. of normal horse serum intracardially; mild anaphylactic symptoms. Guinea Pig 2, 0.5 cc. of normal horse serum intracardially; anaphylactic death in 3 minutes. Guinea Pig 3, 0.5 cc. of normal horse serum intracardially; no anaphylactic symptoms. Guinea Pig 4, 0.5 cc. of normal horse serum intracardially; no anaphylactic symptoms.

*Experiment 7.*—Rabbit 16; weight 2,100 gm. June 15, 17, 18, 19, 21, and 23, 1920. On each of these days a subarachnoid puncture was made, 0.5 cc. of cerebrospinal fluid was withdrawn, and 0.5 cc. of normal horse serum injected.

Rabbit 17; weight 2,040 gm. June 15, 17, 18, 19, 21, and 23, 1920. On each of these days 0.5 cc. of normal horse serum was injected intravenously.

*Precipitin Tests with Sera 16 and 17 against Dilutions of Normal Horse Serum.*

Date.	1: 10		1: 100		1: 500		1: 1,000		1: 5,000		1: 10,000	
	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.
1920												
June 23.....	+	0	++	0	++	0	+	0	≠	0	0	0
" 24.....	++	≠	++	+	++	+	++	+	+	0	+	0
" 25.....	++	+	++	++	++	+	++	+	+	0	+	0

Controls: normal rabbit serum and normal sheep serum against dilutions of normal horse serum, negative.

*Precipitin Tests with Dilutions of Sera 16 and 17 against Serum 15, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Blood.*

Date.	1: 10		1: 100		1: 500		1: 1,000	
	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.	Serum 16.	Serum 17.
1920								
June 21.....	+	++	0	+	0	0	0	0
" 22.....	≠	+	0	≠	0	0	0	0
" 23.....	0	+	0	0	0	0	0	0

<sup>14</sup> These sera given on June 25 were withdrawn just prior to guinea pig inoculations.

*Precipitin Tests with Dilutions of Cerebrospinal Fluid 16 against Serum 15, an Anti-Horse Rabbit Serum, in Order to Detect Unchanged Horse Serum (Precipitinogen) in the Cerebrospinal Fluid.*

Date.	1:10	1:100	1:500	1:1,000
1920				
June 21.....	+	0	0	0
" 22.....	+	0	0	0
" 23.....	Lost.	Lost.	Lost.	Lost.

June 25, 1920, 2.25 p.m. Guinea Pig 5, 0.1 cc. of Rabbit Serum 16 intraperitoneally. Guinea Pig 6, 1 cc. of Rabbit Serum 16 intraperitoneally. Guinea Pig 7, 2 cc. of Rabbit Serum 16 intraperitoneally. Guinea Pig 8, 0.1 cc. of Rabbit Serum 17 intraperitoneally. Guinea Pig 9, 1 cc. of Rabbit Serum 17 intraperitoneally. Guinea Pig 10, 2 cc. of Rabbit Serum 17 intraperitoneally. June 26, 4 p.m. Guinea Pig 5, 0.5 cc. of normal horse serum intracardially; no anaphylactic symptoms. Guinea Pig 6, 0.5 cc. of normal horse serum intracardially; severe anaphylactic symptoms. Guinea Pig 7, 0.5 cc. of normal horse serum intracardially; died in 4 minutes. Guinea Pig 8, 0.5 cc. of normal horse serum intracardially; no anaphylactic symptoms. Guinea Pig 9, 0.5 cc. of normal horse serum intracardially; no anaphylactic symptoms. Guinea Pig 10, 0.5 cc. of normal horse serum intracardially; mild anaphylactic symptoms. Guinea Pig 11 (control; injected May 10 with 1 cc. of normal horse serum intraperitoneally), 0.1 cc. of normal horse serum intracardially; died in 3 minutes.

Text-figs. 1 to 3 represent graphically the precipitin response in all the rabbits. Precipitin tests for unchanged horse serum in the blood and spinal fluid, as well as the strength of all the reactions, are omitted to avoid confusion.

#### DISCUSSION.

The marked discrepancy between precipitin formation in the blood of rabbits following intraspinal and intravenous injections of the precipitinogen, coincides with differences in clinical manifestations after administration of horse serum by these channels. The explanation of the reactions is not clear. Two alternatives are considered: purely mechanical factors whereby horse serum in the cerebrospinal fluid gains access to the blood more slowly than when injected directly into the veins; and a specific influence upon precipitin formation by nervous tissue cells.

In the first instance, a gradual introduction of the precipitinogen into the blood may conceivably have a different stimulating effect upon precipitin formation than when the entire dose is injected intravenously. This may be especially true when serum is repeatedly given, each subsequent intravenous dose tending to combine and thus to use up precipitins already formed. That horse serum injected into the subarachnoid space is detected there 24 and 48 hours after injection may be due to leakage from the blood in spite of the clear character of the cerebrospinal fluid after frequent punctures. Flexner and Amoss<sup>15</sup> have pointed out that meningeal injury capable of causing permeability is not always sufficient to cause morphological changes in the fluid. Mention may be made of experiments by Jacob,<sup>16</sup> who demonstrated iodine and other substances in cells of the central nervous tissues several days after intraspinal administration. In the studies reported here no parallel experiments with intraperitoneal and subcutaneous injections were made. However, it is generally advocated that intravenous administration of the precipitinogen is more effective in precipitin production than the routes mentioned above.

There is little evidence to show that the central nervous system exerts a specific influence on precipitin production and anaphylactic reaction. It is noteworthy, however, that the intracerebral injection of horse serum in sensitized guinea pigs is one of the most rapid and effective methods of producing anaphylactic shock, as pointed out by Besredka and Steinhardt<sup>17</sup> and confirmed by Rosenau and Anderson.<sup>18</sup>

An interesting phase of these experiments is the small amounts of horse serum required to produce potent precipitin formation in contrast to the large doses usually advocated. This experience has recently been confirmed by Mackenzie.<sup>19</sup>

Contrary to expectation, clear cerebrospinal fluid was obtained after repeated intraspinal injections of horse serum which contained no preservative.

<sup>15</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

<sup>16</sup> Jacob, P., *Deutsch. med. Woch.*, 1900, xxvi, 46.

<sup>17</sup> Besredka, A., and Steinhardt, E., *Ann. Inst. Pasteur*, 1907, xxi, 117.

<sup>18</sup> Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H.*, No. 45, 1908.

<sup>19</sup> Mackenzie, G. M., personal communication.

Unfortunately, no cases of meningitis appeared when this subject could be studied clinically. Therefore, whether the clinical reactions noted above are due to a mechanism similar to that in these experiments remains to be proved.

#### SUMMARY.

1. Rabbits which have received a single dose of normal horse serum in the subarachnoid space produce precipitins in the blood in greater abundance, of higher titer, and persisting longer than those in control rabbits which have received a similar injection intravenously.

2. Repeated subarachnoid injections of normal horse serum in rabbits induce precipitins in the blood early. These may appear in high titer as soon as 1 week after the initial injection, whereas in rabbits similarly treated intravenously no precipitins are found at this time. They may appear a few days afterward and reach a high titer.

3. No anaphylactic manifestations occurred in rabbits treated repeatedly with subarachnoid injections of normal horse serum when the precipitin content of the blood was high.

4. Anaphylactins, as determined by passive transfer of anaphylaxis, were demonstrated in sera with high precipitin content.

5. These experiments may explain clinical evidences of anaphylaxis, observed when an initial intravenous injection of horse serum followed a series of intraspinal injections of such serum.

# GRANULES IN THE CELLS OF CHICK EMBRYOS PRODUCED BY EGG ALBUMIN IN THE MEDIUM OF TISSUE CULTURES.

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PLATE 51.

(Received for publication, January 4, 1921.)

It has been shown that connective tissue cells of the chick embryo become greatly altered when placed in an abnormal environment (M. R. Lewis, 1918; W. H. Lewis, 1919; M. R. Lewis, 1920; Prigosen, 1921). These alterations in appearance can be compared with certain changes which take place in pathological conditions of adult tissue such as fatty degeneration, necrosis, autolysis, phagocytosis, etc.

The present paper is a report of the results obtained when white of egg is introduced into the medium of a tissue culture of connective tissue. This substance not only has a decidedly toxic influence upon the growth of the cells, but it also produces a marked change in the appearance of the cells comprising the growth, owing to an accumulation of large, somewhat refractive granules within the cytoplasm of these cells. These granules take the eosin stain when methylene blue and eosin are used. In this regard the connective tissue cells resemble the epithelium of renal tubules filled with colloid or hyaline droplets described by MacCallum (1916).<sup>1</sup> On the other hand, when the stain used is iron-hematoxylin, these cells closely resemble certain cells undergoing active secretion described by Hoven (1910), Mislawsky (1913), and Saguchi (1920).

It has been shown that explants of connective tissue from the chick embryo grow in pure egg albumin (Swezy, 1915). These cultures do not live so long or attain so extensive a growth as cultures explanted in Locke-Lewis solution. Cells from the subcutaneous tissue

<sup>1</sup> MacCallum (1916), p. 91.

of the chick embryo, explanted into egg albumin, become filled with large round granules of different sizes (Figs. 1 and 2). It has not been possible so far to demonstrate the exact chemical nature of these granules; therefore, in order to differentiate them from other granules observed in the cells, they will be called al. granules throughout this paper. This term is not meant to signify that these granules are or are not albumin, but only to specify that they are due to the presence of albumin in the environment of the cells.

*Chick Embryo Connective Tissue in Egg Albumin.*

An egg was opened under sterile conditions and a small quantity of the white removed to a sterile dish. This was then cut many times with sharp curved scissors in order to obtain a solution of egg albumin. A piece of connective tissue was removed from a chick embryo of 7 to 10 days incubation and placed on the solution of egg white where it was cut up into small pieces. Each piece, with a small quantity of egg albumin, was placed on a clean cover-glass and inverted over a vaseline ring on a depression slide. After 16 to 20 hours connective tissue cells had grown out into the medium in the same manner as in normal cultures, though by no means in such great numbers or to so great an extent. All these cells contained a number of rather large granules, usually round in outline. The granules were composed of a substance which differed in appearance from the cytoplasm. They seemed to be denser, more opaque, and possessed an index of refraction different from that of the cytoplasm. In fixed preparations they stained a much deeper tone than the cytoplasm, becoming black and gray with hematoxylin but never so pale as the cytoplasm. There was great variation in the size of the granules in a given cell, as well as in those in different cells. They were in most cases much larger than either the mitochondrial bodies or the neutral red granules.

Shipley (1919)<sup>2</sup> described somewhat similar granules in plasma cultures of chick embryos. He stated, however,<sup>3</sup> that the degeneration vacuoles described by Lewis and Lewis were identical with these

<sup>2</sup> Shipley (1919), p. 288.

<sup>3</sup> Shipley (1919), p. 289.

plasma granules, and that the differences in the two bodies were due to differences in the media. This is not true in regard to the al. granules characteristic of the albumin cultures, for the two bodies, *i.e.* the al. granule and the degeneration vacuole (W. H. Lewis, 1919), may exist side by side in degenerating cultures (Figs. 3 and 5). In such cultures the al. granules were markedly different from the degeneration vacuoles in appearance and were easily distinguishable from them, not only in the living cell but also in fixed material.

Plato (1900) injected dried and powdered white of egg into the abdominal cavity of a guinea pig. Shortly afterwards leucocytes were obtained from this region and stained with neutral red. They were found to contain large, irregularly shaped, orange-red clumps, which Plato supposed were ingested particles of white of egg taken in as foreign bodies. The structures obtained in this manner do not resemble to any great extent those described above as al. granules.

After 24 to 48 hours a difference could sometimes be detected in the appearance of the individual al. granules in a given cell. Some appeared to be of a different consistency, but whether this could be expressed as more fluid is doubtful. In the stained preparations certain of the granules were much darker than others (Fig. 2) and the changed, possibly more fluid, granules of the living cell probably correspond to the more lightly stained ones in fixed cultures.

The al. granules differed from certain other types of granules in living cells, such as pigment granules, neutral red granules, and certain secretion granules, in that they were less frequently found collected around the centrosphere (Figs. 2 and 5) than in other regions of the cytoplasm. The processes of the cells were always free from al. granules. Frequently there was an extensive ectoplasm which the granules did not enter, but occasionally this was reduced to a narrow ectosarc on one or both sides of the nucleus. These granules obscured the other granules in the living cell, except at the periphery, where the mitochondria could be seen extending out into processes of the cell. Spindle-shaped cells (Fig. 1) were more numerous than were the flat cells (Fig. 2). These elongated cells contained granules in the neighborhood of the nucleus but there were none in the ends of the cells. The cells divided by mitosis, even when full of granules (Fig. 4). This is another means of differentiating these granules from

the degeneration vacuoles, as cells containing vacuoles rarely undergo mitotic division.

As a rule, all the connective tissue cells of the growth contained some al. granules. Certain other kinds of cells in the same culture, however, did not have these granules. While the number of these bodies in a given connective tissue cell increased to some extent with the age of the cultures, most of the cells contained the maximum number at the end of 24 hours.

It was not possible to observe the fate of the granules in cultures grown in egg albumin, because degeneration took place too rapidly. Generally, after 48 hours, degeneration vacuoles began to appear. These signs of degeneration took place first in the cells along the periphery of the growth. In most of the flat cells the cytoplasm was sufficiently spread out so that it could be readily seen that the region of the centrosphere was free from al. granules and that a number of degeneration vacuoles had collected there (Figs. 3 and 5). From the location of the degeneration vacuoles it is evident that they were not formed from al. granules which had become fluid due to digestion of the substance forming them. After 72 hours vacuoles were present between some of the al. granules, as well as in the region of the centrosphere (Fig. 5), and many of the cells were so degenerate that it was impossible to determine whether the vacuoles were around the granules or between them. In some of the degenerate cultures the al. granules were small and fragmented. After the vacuoles appeared the cells rapidly assumed a rounded form so that the details of the phenomenon could not be observed clearly. The vacuoles increased in number, and degeneration took place much more rapidly than in the normal cultures. Few of the cultures in egg albumin survived longer than 3 or 4 days, while most of the control cultures grown in Locke-Lewis solution lived for over 2 weeks.

Whether the albumin was taken from a freshly laid or from an incubated egg made no difference in the appearance of the al. granules, although the hydrogen ion concentration of an incubated egg differs from that of an unincubated egg. The cells of cultures made with albumin from an incubated egg also divided by mitosis (Fig. 9) and produced a small growth around the explant. After a few days, degeneration took place and the cells died about as rapidly as in albumin from the fresh egg.



*Cultures in Diluted Egg Albumin.*

Much the same result in regard to the accumulation of granules in the cells was produced when the albumin was diluted one-half with Locke-Lewis solution; but when the quantity of albumin was greatly reduced the results became less constant. This may have been partly due to the difficulty of obtaining a uniform solution of the egg albumin. In media containing only a small percentage of egg albumin (1 to 5 per cent) many of the cultures exhibited no accumulation of granules in the cells (Figs. 6 and 7); in others a few of the cells contained many granules, the remaining cells being entirely free from them; while in still other cultures a few small granules were present in every cell. Some cultures in diluted egg albumin remained normal for several days but later a number of the cells exhibited typical al. granules. The smaller the percentage of albumin in the medium, the more nearly normal did the cultures appear in extent of growth and length of life; in none of the percentages of albumin used did the growths become so large or live so long as they did in the Locke-Lewis solution.

*Changes Produced in Cultures When the Locke-Lewis Solution Is Replaced by Egg Albumin.*

The hanging drop was removed from 24 to 48 hour cultures of chick connective tissue which had been explanted in Locke-Lewis solution and was replaced by egg albumin. This caused immediate changes in the appearance of the normal connective tissue cells (Fig. 11), due not only to the change in the nature of the environment from a neutral or slightly acid fluid to a markedly alkaline jelly, but also to the manipulation. The mitochondria usually assumed the form of short rods and granules instead of filaments, and the cells became somewhat rounded, showing a number of processes. In some instances many of the cells died. Al. granules did not appear for several hours, but after 20 hours these bodies were about as abundant and of the same size as those in cells of cultures explanted directly into egg albumin. It was not observed that the mitochondria stored up albumin and changed into the al. granules. They did, however, become shorter and seemed to decrease

in number as the cells became full of granules. The size of the growth of cultures under these conditions was always larger than that of cultures explanted directly into egg albumin, because the growth had already become extensive in the normal medium before the egg albumin was placed upon it. When these cultures degenerated, vacuoles appeared in the cytoplasm in the usual manner, regardless of the number or size of al. granules in the cells. Some of the cells degenerating under these conditions presented unusual appearances, especially in the region of the centrosphere. In some instances large bodies resembling a certain type of giant centrosphere described by W. H. Lewis (1920) were observed (Fig. 12).

*Effect Produced by Vital Dyes.*

When a solution of Janus green was placed upon a culture the cells of which contained al. granules, the mitochondria became a bright blue-green, while the al. granules remained unstained. After the cells began to die, as they soon did owing to the toxic effect of the stain, the al. granules sometimes took on the green color. There were fewer mitochondria in cells living in egg albumin than in those in Locke-Lewis solution and the filaments extending out into the processes were shorter. Scattered among the al. granules were a few, short, rod-shaped and granular mitochondria. In the region of the centrosphere the mitochondria were more like those of normal cultures.

Trypan blue dissolved in Locke-Lewis solution, when placed upon the cultures, failed to stain any body in the cell until a number of hours had elapsed; then a few small blue granules appeared but the al. granules remained colorless (Fig. 8).

Neutral red was taken up by the vacuoles and granules in these cells in about the same manner as in the normal cultures. After a few hours the al. granules were sometimes unstained, again they appeared to be pale pink, and in other cases they seemed to be outlined with red.

*Fixed and Stained Preparations.*

The al. granules were more successfully fixed and stained than were the mitochondria. Methods of fixation which did not preserve the mitochondria frequently afforded good results in as far as the al. granules were concerned. An easy method of fixation by which both types of granules are preserved is to wash off the albumin by means of warm Locke-Lewis solution and drop the cover-slip into Zenker's solution from which acetic acid has been omitted. With iron-hematoxylin the al. granules stained in various shades, from dense black to gray, the result probably depending upon the concentration of the material forming the granule. In appearance the cells bear a striking resemblance to the secretion cells illustrated by Hoven (1910), Saguchi (1920), Mislawsky (1913), and others. All the forms described by these investigators to show stages in the change from mitochondria into secretion granules can be found. This suggests that Scott (1916) probably was correct in his view that the granules present in certain gland cells may be material accumulated in the cytoplasm and not necessarily formed directly from any preexisting structure of the cell, such as the mitochondria.

*Cultures of Fish Embryos in Egg Albumin.*

A few cultures of embryos of *Fundulus heteroclitus* were made in egg albumin. The cells of these cultures grew much more slowly but almost as extensively in the egg albumin as did those in the fluid media described by Dederer.<sup>4</sup> Instead of all the mesenchyme cells becoming filled with al. granules, as they do in chick cultures, only a few along the edge of the growth showed the granules. This may be due to the fact, as shown by Dederer, that the mesenchyme cells grow along the cover-slip and are in most places covered by a layer of ectoderm cells which separate them from the medium. The ectoderm did not form al. granules to any appreciable extent. The peripheral mesenchyme cells, which contained al. granules (Fig. 10), appeared much the same as did those of the chick embryo.

<sup>4</sup> Dederer, P. H., personal communication.

## DISCUSSION AND SUMMARY.

It is difficult to understand what factors may be concerned in the formation of the al. granules. The phenomenon may be concerned with changes in the cell membrane due to an abnormal environment; that is, material which would otherwise be excluded may be permitted to enter the cell, or, on the other hand, certain substances may be prevented from passing out of the cells. Previous investigators have shown that mesenchyme cells sometimes engulf certain foreign bodies, and it is possible that the solution of white of egg is ingested in the same manner. When a solution of peptone was placed on the cells instead of egg white, the phenomenon did not occur (Fig. 13); the cell remained normal and degenerated in the usual manner (Fig. 14). This would seem to indicate that the al. granules are not formed from peptone. Regardless of the factors involved, it is evident that egg albumin in the medium of tissue cultures of chick embryos causes the formation of numerous large granules in the cytoplasm of the connective tissue cells. This phenomenon is associated with unfavorable conditions for the life of the cells and results in the rapid death of the cultures.

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## EXPLANATION OF PLATE 51.

All the figures are camera lucida drawings of cells from the subcutaneous tissue of chick embryos; oil immersion lens and No. 6 ocular.

FIG. 1. A spindle cell containing al. granules from a 48 hour culture of an 8 day embryo in egg albumin.

FIG. 2. A flat cell containing al. granules from a 48 hour culture of a 7 day embryo in egg albumin.

FIG. 3. A flat cell containing al. granules; a few degeneration vacuoles may be seen in the region of the centrosphere. 48 hour culture of an 8 day embryo in egg albumin.

FIG. 4. Mitosis of a cell containing al. granules.

FIG. 5. A cell from a 24 hour normal culture which had egg albumin on it for 72 hours.

FIG. 6. A cell from a 4 day culture in diluted egg albumin. This cell does not contain al. granules.

FIG. 7. A degenerating cell from a 4 day culture in diluted egg albumin.

FIG. 8. A cell from a culture of an 8 day embryo in egg albumin which had been stained with trypan blue.

FIG. 9. A cell undergoing mitotic division from a culture of an 8 day embryo in albumin from an egg incubated for 12 days.

FIG. 10. A cell from a culture of *Fundulus* embryo in egg albumin.

FIG. 11. A cell from a 10 day culture of an 8 day embryo in Locke-Lewis solution.

FIG. 12. A cell containing a giant centrosphere from a culture which had the Locke-Lewis solution replaced by egg albumin for 24 hours.

FIG. 13. A cell free from al. granules which had 1 per cent Bacto peptone on it for 24 hours.

FIG. 14. A cell from a culture which had 5 per cent Bacto peptone placed upon it. This cell contains no al. granules but has degeneration vacuoles.





(Lewis: Tissue culture of chick embryos.)





## EXPERIMENTAL SYPHILIS IN THE RABBIT.

### VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

#### PART 1. LESIONS OF THE SKELETAL SYSTEM.

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PLATES 52 TO 60.

(Received for publication, December 27, 1920.)

Syphilitic lesions of the skeletal system of the rabbit were first reported by Uhlenhuth and Mulzer (1) in 1910 under the rather indefinite term of "nose tumors." The conditions described by them were produced by intracardial and intravenous injections of massive doses of *Treponema pallidum* in young rabbits and appear to have been chiefly tumor-like swellings of the nasal mucosa and the soft tissues about the end of the nose. However, periostitis was described in one instance, and subsequent reports (2) contained illustrations of several types of the "nose tumors," which were obviously lesions of the nasal bones and cartilages.

Reasoner (3) also has mentioned the occurrence of a periosteal lesion of the nasal bones and refers to this type of affection as a late manifestation of the infection produced by certain strains of *Treponema pallidum*. None of these authors indicates the relative incidence of this class of lesions, and these are the only references to lesions of the skeletal system which have come to our notice. Apparently no instance of such a condition has been reported following a local inoculation, unless it be the case referred to by Reasoner.

In our experience, however, localized infections of the skeletal system, including the bones of the feet and legs and face, were quite common, hence it seems that the conditions have passed unrecognized and are, therefore, essentially new additions to the subject of experimental syphilis.

Among our earlier animals, there were 33, or 26 per cent, of those showing manifestations of generalized syphilis, in which lesions of the periosteum, bone, cartilage, tendons, and tendon sheaths were recognized by simple palpation and inspection. With the introduc-

tion of methods intended to increase the incidence and the severity of the generalized infection, as indicated in a previous paper (4), there was a marked increase in this particular group of conditions and the total number of cases available for study was more than doubled. In addition, the use of the radiograph in the diagnosis and study of deep seated lesions has been of considerable advantage.

The majority of the lesions seen were instances of periosteal, perichondrial, or bone involvement; only a few cases were recognized clinically in which the lesions appeared to originate in tendons, tendon sheaths, and like structures, and in a few instances affections of the joints were noted which appeared to be attributable to syphilitic infection. The parts chiefly affected were the facial and cranial bones and cartilages, and the bones, tendons, and joints of the feet, legs, and tail.

All these are lesions of hidden parts and in order to convey some idea of the nature of the processes concerned, it will be necessary to preface the clinical description of this form of experimental syphilis with a brief description of the lesions themselves.

#### *General Character of the Lesions.*

Lesions of the skeletal system proper occurred both in the periosteum and in the deeper parts of the bone or cartilage but most of the cases which came under our observation were clearly instances of primary periosteal involvement.

*Periosteal Lesions.*—These were of two types, the more common one being a nodular, granulomatous condition and the other a process of a more diffuse character. The first was characterized by the formation of small flattened or oval plaques, or of elevated nodules of extreme hardness distributed over the surface of the bones. Some of them spread laterally, forming masses which could barely be detected, while others became more elevated and reached a size of from a few millimeters to more than a centimeter in diameter.

Clinically, these lesions were readily distinguishable from cutaneous affections by the fact that the skin was freely movable over them and they were firmly attached to the underlying bone. When exposed to view, the earlier lesions appeared translucent and of a pale, slightly

yellow color, or presented an opalescent appearance and were surrounded by a faint areola of newly formed vessels. As the lesions developed, they became more dense and changed to a gray or yellowish gray color. Some were highly vascular and showed irregularly distributed areas of congestion and hemorrhage together with foci of necrosis. These conditions may be illustrated in general by Fig. 1, which is a photograph of an autopsy specimen showing the condition present 11 days after the lesions were first noted.

Upon section, some of the lesions were dense and fibrous, while others were more succulent or of a fleshy character. The cut surface of the larger ones frequently presented a mottled appearance due to the presence of irregularly distributed areas of necrosis and hemorrhage. In other cases, the surface was thickly stippled with minute gray or yellow points of necrosis or the entire center of the lesion was necrotic and demarcated from an outer zone of living tissue by the presence of a gray or yellowish gray line (Figs. 2 and 3).

The necrotic portion of the lesion remained firm and elastic, as a rule, but in exceptional instances or in cases of extensive necrosis, the central portion broke down into a softened or a cheesy mass (Fig. 3).

At the beginning, lesions of this type were largely confined to the periosteum, but as the process advanced, they frequently extended to the underlying bone. The changes in the bones themselves were quite variable. In general, they consisted of erosion of the outer table, or of bony caries, and finally led to an osseous overgrowth or the formation of osteophytic nodes. The destructive changes are shown in Figs. 4 and 5.

The first photograph (Fig. 4) shows the condition found at autopsy in an animal with actively progressing lesions of the facial bones. There were three groups of lesions present, the main mass occupying a position over the bridge of the nose while smaller lesions were present on both sides at a slightly higher level.

Upon turning back the flap of periosteum covering the left side of the nose (Fig. 5), the upper lesion appeared as a thickening of the periosteum corresponding with an area of erosion in the bone beneath. Over the bridge of the nose, there was a rather large granulomatous mass, most of which was firmly united with the periosteum. In the position occupied by this mass, a considerable area of the nasal bone had been completely destroyed, exposing the periosteum of the under side of the bone which was also affected. These lesions had been recognizable for only 19 days and yet the degree of destruction was quite marked.

Pathologically, the diffuse periostitis differed from the more circumscribed or nodular form chiefly in the presence of a thin layer of rather soft and friable tissue which covered a wide area of bone. During the earlier stages of the infection, little or no alteration could be detected in the contour of the part, but as the lesions increased, slight thickenings or irregularities could be made out.

On the whole, this type of process appeared to be more destructive than the circumscribed or nodular form; that is, its extension was lateral and inward rather than upward or outward. These lesions also tended to produce a chronic fibrous thickening of the periosteum such as that shown in Figs. 6 and 7. The thickening of the periosteum is here most apparent near the end of the nose where a bulbous enlargement has been produced. This was due in part to periosteal thickening and in part to a thickening of the bone and nasal cartilages (Fig. 7).

With bones such as the nasals, periosteal lesions developed from the under side of the bone as well as from the outer side. This is shown in a low power magnification of a cross-section of the nasal bone reproduced in Fig. 8.

Perichondrial lesions, especially those about the face, presented essentially the same characteristics as those of the periosteum. During periods of active growth, spirochetes could always be demonstrated in these as well as other bone lesions to be described, and were usually present in very large numbers.

*Lesions in the Bone and Marrow Cavities.*—In addition to primary periosteal involvement with subsequent extension to the bone, a number of lesions were seen which originated within the bone or marrow cavities. This class of infections, including what might be designated as osteitis, osteomyelitis, and epiphysitis or osteochondritis, is still somewhat obscure owing to the difficulty in detecting the lesions at an early stage of their development.

Clinically, little could be told of these affections by ordinary methods of examination until they had reached a fairly advanced stage. Necrosis of such bones as the nasals could then be detected by a crackling or giving of the bone beneath the palpating finger, and as the process extended to the outer surface of the bone, a deformity was produced which in most instances presented the same picture as that

of a primary periostitis. Lesions at the epiphyses of the long bones could also be detected in some instances by the presence of a swelling at the epiphyseal line, but they were difficult to distinguish from periosteal lesions which showed a marked tendency to localize at the same points.

By the use of x-rays, some early lesions were detected and their development was followed, but, as a rule, infection of the deeper parts of the bone was not discovered until the picture had been complicated by necrosis with dissolution of the bone or by pathological fracture. There are, therefore, two groups of conditions to be considered—one including lesions of obvious syphilitic origin and the other affections of a more obscure character.

By radiographs and by pathological examination, lesions were found in both the long and the flat bones. These appeared as focalized processes composed of a pale, translucent, and almost gelatinous material which spread out in the marrow cavities to a greater or less extent and invaded the substance of the bone. A lesion of this type is shown in Fig. 9 which is taken from the so called fifth metatarsal. As may be seen, there were in this case both periosteal and endosteal lesions as well as focal lesions within the bone itself. That the periosteal and endosteal lesions were not parts of the same process could be determined by differences in their histology, but such distinctions could not be made with lesions within the bone.

The internal lesions appeared to arise chiefly from the membrane lining the marrow cavity (endosteum) or in the loose perivascular tissues surrounding the larger vessels. In the long bones, they also showed a predilection for lines of epiphyseal union as shown in Fig. 10. This represents a fully developed lesion which originated in the epiphysis and subsequently spread to other parts of the bone.

Radiographs of the early lesions showed mainly a rarefaction in the bone and a loss of the finer details of structure. These changes may be seen in Fig. 11, which shows two very distinct areas of involvement. One of these is in the shaft of the fifth metatarsal of the right foot (*a*) and the other at the proximal end of the corresponding bone of the left foot (*b*). The deformity of the right tarsus and their regularity seen in the calcaneus (*c*) were the result of an older lesion of a type to be described later.

This class of infections naturally gave rise to pathological alterations in the bone, which were characterized by necrosis with a more or less gradual disintegration of the bone or by increased fragility (when necrosis was more rapid) and the occurrence of pathological fracture or epiphyseal separation.

With these facts in mind, attention may be called to another group of bone lesions whose early stages are very difficult of recognition. These include chiefly cases of necrosis, fracture, and epiphyseal separation of the calcaneus with, occasionally, similar lesions in other bones, and may be illustrated by the affections of the tarsus shown in Figs. 12 to 14.

The history of these affections was much the same in all cases; namely, the sudden development of lameness, with a marked edematous swelling of the entire tarsus together with some tenderness and the presence of a crepitus. The first cases which were noticed were regarded as traumatic injuries and no particular attention was paid to them until it was found that similar conditions occurred as a result of obvious syphilitic infection.

A more careful investigation was then attempted and the nature of the injury to the bone determined by both radiographic and pathological examination. Spirochetes could not be found in fluid aspirated from these cases<sup>1</sup> and the condition was complicated by the reaction incident to the sudden disruption of the bone.

From an examination of serial radiographs of several of these animals, abnormalities of the bones could be made out in some which antedated the occurrence of the lesion in question. In Fig. 15, a peculiar defect is seen in the calcaneus of the right foot. 19 days later, this bone gave way in the middle and anterior portions, permitting the talus to sink downward as shown in Fig. 16, the details of the bony structures being masked at this time by an effusion into the tissues. There was also a dropping of the arch.

A second case is shown in Figs. 17 and 18. The first point to be noted is the dissimilarity of the posterior ends of the calcanei. The left bone shows a narrowing of the neck which extends well below the level of the tuberosity, while on the right, there is even some fullness in this region. This bone shows a band of lighter

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<sup>1</sup> The demonstration of spirochetes in such cases by animal inoculation would be of no value, since the animals were known to have a generalized infection capable of transmission by blood inoculation.

shadow in the position of the epiphyseal line. 8 days later (Fig. 18) there was an epiphyseal separation, or fracture, which followed this line quite closely. Other examples of a similar character were also seen.

A significant feature of this class of lesions is that among the large number of animals, infected and uninfected, which were handled during the course of this work, with one possible exception, lesions of this type occurred only in animals with obvious syphilitic lesions of other bones (note metatarsals in Figs. 11 and 12). It may be mentioned also that definite periosteal lesions of the anterior end of the calcaneus have been noted, and while no gross destruction of bone was detected in these instances, all the obscure cases of necrosis such as that in Fig. 12 have occurred in exactly the same position as that occupied by the periosteal lesions.

Histologically, however, the extent of bone involvement always proved to be greater than the gross appearance would indicate, and bones which appeared to be very little affected in the gross frequently proved upon histological examination to be honeycombed by the syphilitic process. Further description of these lesions will be found below in the section on the histological changes observed in various types of bone lesions.

*Lesions of Tendons and Tendon Sheaths.*—There is no record of the occurrence of syphilitic lesions of the tendons or tendon sheaths of the rabbit so far as we are aware, and the number observed by us was comparatively small.

The typical lesion in cases of primary tendon involvement was a circumscribed granulomatous process of essentially the same character as that of the periosteum, and with a few exceptions the lesions were located in the tendo achillis or its sheath. The exceptions noted were small multiple lesions involving the tendons on the dorsum of the carpus and front feet and the dorsum of the hind feet. Some of these were not recognized until the animal came to autopsy, and it is possible that other cases of a similar character might have escaped our notice.

Secondary involvement of tendons and ligaments as a result of direct extension of lesions originating elsewhere was of comparatively frequent occurrence. This was not unusual with periosteal lesions

about the carpus and tarsus and the small bones of the feet, and with cutaneous lesions of the tail and lateral surfaces of the hind feet. Fig. 2 furnishes a good illustration of this type of condition.

*Diffuse Exudative Reactions Associated with Lesions of the Bones.*

In addition to the lesions described, an acute exudative reaction affecting the parts immediately surrounding a focus of bone affection may be referred to briefly. This reaction rarely occurred except with lesions of the tarsus or of the small bones of the feet. In cases of infection of the metatarsals and the phalanges, there was frequently an exudation into the surrounding tissues which was composed chiefly of serum with a few leucocytes and polyblasts. Such reactions occurred most often where there was a considerable degree of bone destruction.

Similar conditions have already been referred to in connection with lesions of the tarsal bones. In addition, there were a few instances in which the reaction occurred where no bone lesion was demonstrable. The inciting factor in these cases is not known, but the subsequent history of the condition, with the development of a diffuse fibrosis and fixation of the affected parts, suggests the possibility of an involvement of synovial membranes of tendon sheaths and joint cavities. Histologically, it has been shown that the *pallidum* infection may become localized in such structures, but whether this takes place by direct extension from other lesions or as an independent focus of infection is as yet undetermined.

*Histology.*

Histologically, the details of the pathological process concerned in syphilitic infections of the bones are too numerous and too complex to be described in the present connection. As a means of orientation, however, it seems well to refer briefly to certain features of the lesions found during active stages of the infection. Three fairly well defined groups of conditions can be recognized: (1) a granulomatous process which is not unlike that of other syphilitic lesions; (2) a condition which is characterized chiefly by absorption or by necrosis and disintegration of bone; and (3) a lesion which combines the processes



of granulomatous proliferation, absorption, and necrosis with osseous overgrowth. This last condition will not be considered at the present time.

The granulomatous lesions usually seen in cases of bone syphilis may arise from any part of the bone and present certain structural peculiarities. In general, they are composed of a matrix of newly formed connective tissue with a network of capillary vessels; there are the usual polyblastic infiltration and a marked tendency on the part of these cells to assume a perivascular arrangement. Polynuclear giant cells are also present and are frequently very numerous, especially where marked bone destruction occurs. The picture presented in these cases is of the type of an active foreign body reaction or may even suggest that of the so called giant cell osteosarcoma.

When the lesion arises from the periosteum, one frequently finds an interesting structural arrangement of the elements which participate in the reaction (Figs. 19 and 20). There are two or three distinct layers which correspond roughly with structural divisions of the periosteum. There is first an outer cellular layer which is composed of loosely arranged connective tissue cells and polyblasts occupying the position of the loose areolar tissue covering the outer surface of the periosteum (Figs. 19 and 20). Then comes a denser layer of fibroblasts which contains numerous focal and perivascular accumulations of round cells and corresponds to the dense fibrous layer of the periosteum. Finally, either as a part of this second stratum or as a distinct layer in itself, there is a zone which corresponds with the osteogenetic layer of the periosteum. At first, this is composed of a row of giant cells (osteoclasts) whose processes dip downward into the bone, and a small amount of newly formed connective tissue infiltrated with polyblastic cells. This is the osteoclastic layer of the lesion and its distinctive elements, the osteoclasts, are derived from the osteoblasts of the periosteum.

The composition of periosteal lesions differs very decidedly in different classes of bones. Thus, in bones which are preformed in membrane, such as the nasal bones, a two layered structure prevails (Fig. 19). This is composed of the loose outer layer and a highly developed third or osteoclastic layer, while the second or fibrous layer is usually absent or but slightly developed. The lesions of the long bones

(cartilage bones), on the other hand, show three layers, the most highly developed of which is the middle or fibrous layer. In addition, the osteoclastic layer is more fibrous and shows relatively few giant cells. These facts are of importance in interpreting the pathological alterations produced in different classes of bones.

The growth of some periosteal lesions is marked by an increase in the thickness of the outer layers, of others by a peripheral extension of all three layers, or by a downward growth of the inner layer. In the first instance, the condition produced is a prominent periosteal nodule, or granuloma, in the second a diffuse periostitis, and in the third marked destruction of the underlying bone. It is in instances of the last group that the osteoclastic layer of the lesion becomes so highly developed; in membrane bones, the greater part of the lesion in such cases presents the picture of the giant cell granuloma, but this is not true of bones which are preformed in cartilage.

Lesions arising within the bone show essentially the same composition as those from the periosteum, except for their structural arrangement, and are, therefore, difficult to distinguish from them. Endosteal affections, on the other hand, are almost devoid of fixed connective tissue elements. They are composed of a loosely arranged stroma which is thickly infiltrated with polyblasts and contains a network of capillary vessels. The lesion is, therefore, more of an infiltrative than of a granulomatous process.

The alterations observed in the bone itself were quite variable. In some instances, the bone presented a practically normal appearance except for the presence of irregularities or a honeycombing of the bone due to gradual absorption, and in some, even small fragments of bone showed a well preserved architecture (Fig. 21). Frequently, however, there were mass necrosis and rapid disintegration of bone extending over wide areas. The picture presented in these cases (Fig. 22) was very striking and consisted of necrosis of bone corpuscles and reduction of the ground substance to a thin and ragged framework of granular or fibrillated material which stained very faintly if at all. The impression created was that of large masses of bone undergoing solution, and various stages in the process could be traced down to the last shadowy framework of fibers and granules (Fig. 22).

Emphasis may be placed upon these processes of bone destruction on account of the occurrence of similar changes in portions of the bone where little or no syphilitic reaction of the generally accepted type could be made out. This was especially noticeable in the lesions of the calcaneus described above. There were two forms of alteration to be noted. In one group of cases, the alteration consisted of a simple bone absorption with reduction in the density and the volume of the bone; in a second, there were necrosis and disintegration.

The first group developed by widening out of the lacunæ and of the perivascular spaces, which became filled with a loose and rather cellular connective tissue. The compact portion of long bones such as metatarsals and phalanges thus tended to become somewhat cancellous in structure, while the cancellous portion of the bone was distinctly reduced in amount. As a result of these changes, there were a reduction in both the density and the thickness of the bones and a marked enlargement of the marrow cavity. Normally, these bones contain some cellular marrow, but in affections of the type described, there were not only bone absorption but a conversion of a cellular (hemopoietic) to a fatty marrow which, in some cases, was complete.

Mass necrosis of portions of the bone which were apparently not directly involved by the granulomatous lesions needs no further description than that given above.

The most obvious explanation of these conditions is one based upon disturbed nutrition. There is little doubt that the primary seat of injury in all syphilitic processes is the endothelial cell, and while those in the immediate vicinity of active syphilitic lesions suffer more than do others, it appears that the effects of the toxic products which are elaborated may reach every such element of the body. Thus, while the smaller vessels in the immediate vicinity of a lesion are most affected, it would seem practically certain that in pronounced cases of syphilitic infection, the entire capillary mechanism is subjected to injury the degree of which varies with the individual case.

If this is applied to the bone lesions described, both the absorption type of phenomenon and the mass necrosis might be accounted for upon the basis of a reduction in the blood supply due to capillary injury in the first case and to a more extensive vascular injury in the second. It would be going too far to assume, however, that other

factors had no part in the production of these lesions, but the reduction of blood supply due to vascular injury appears to be the simplest and most immediate explanation which can be offered.

The fact that rarefaction and necrosis of bone are not necessarily parallel with the size or extent of the granulomatous lesion but may occur even in the absence of any marked granulomatous reaction is of considerable importance, and the obscure cases of necrosis and fracture of the calcaneus described above were largely due to this type of bone lesion.

Histologically, small granulomatous lesions which resemble those of the periosteum or endosteum in all essential respects have been found in the synovial membranes of joint cavities, notably those of the tarsus and the metatarsophalangeal joints. In these cases, there was infection of neighboring parts, and involvement of the joints appeared to have taken place by extension from adjacent foci of infection. While it appears probable, therefore, that primary localization in the joint may occur, this has not as yet been demonstrated.

#### *Gross Alterations in the Bones.*

Frequent reference has been made above to various types of bone injury resulting from infection of the periosteum or of the bone itself. Before concluding the description of this group of lesions, it seems well, however, to give a more definite statement as to the gross alterations which are produced.

*Nasal Bones and Splints.*—The most marked and characteristic lesions were those of the nasal bones and splints. The effects produced may be illustrated by the series of photographs reproduced in Figs. 23 to 30. These photographs were taken from dried skulls and are intended to show alterations produced by focal and diffuse lesions during active stages of the infection as well as the results of repair.

Focal lesions of the nasal bones, as shown in Figs. 23 to 26, may produce anything from the slightest surface erosion or roughening to a complete and sharply circumscribed defect in the bone. All of the group here shown were taken toward the end of the infection and may be regarded, therefore, as representing the maximum of bone destruction produced in each instance. In Fig. 23, there was only a surface erosion, while in the second animal of the series (Figs. 24 and 25) the

necrosis extended much deeper, forming a partial defect in the bone. This defect was easily recognizable during life, and at autopsy it was found that there was little left of the bone except a thin layer of spongy necrotic fragments loosely attached to the underlying membranes. The effect produced in this case might be compared to the saddle-nose deformity of man.

The lesion involving the upper right nasal and the maxillary process of the frontal bone in Fig. 26 shows complete necrosis and removal of the bone, resulting in the formation of a small but very sharply circumscribed defect. It should be stated that the clean-cut appearance presented by this lesion was probably due to the fact that it was of internal rather than external origin. There was a very slight external growth in this case, and when this first appeared, necrosis of the bone had already taken place.

The conditions represented in Figs. 27 and 28 differ from those preceding only in that they represent processes of a more diffuse character, which, as has been explained, are usually more destructive in their effects.

Figs. 29 and 30 represent processes of repair. The lesion in Fig. 29 was of the same type as that in Figs. 27 and 28, but led to complete necrosis of a large part of both nasal bones. The condition here shown is the regeneration effected during a period of about 7 weeks. The bones were considerably thickened throughout; in the upper portion, regeneration was very imperfect, and while the bone towards the end of the nose was of a decidedly spongy character, it was much more nearly normal than that above and the probability is that in time the entire area would have assumed this appearance.

This case illustrates a remarkable tendency on the part of the rabbit to an obliteration of the marks of syphilitic lesions in the bones. Small erosions and defects of the nasal bones are usually repaired so as to leave little if any evidence of the previous injury, and the permanent alterations produced by the most destructive lesions are much less than one might expect. This evidence usually consists of a thickened and adherent periosteum, slight irregularities in the bone, surface roughening and thickening of the bone (*cf.* Figs. 6 and 7), and the presence of slight depressions or bony prominences. The last conditions are occasionally fairly well marked, as indicated by the node on the nasal splint in Fig. 30.

*Anomalous Conditions of the Parietals and Occipitals.*—Before leaving this phase of bone syphilis, attention may be called to the condition of the parietal and occipital bones shown in Fig. 31. The cause of this condition is not known, but, as may be seen, there is a decided thickening of the bones which are very porous or spongy in character.

Various degrees of this condition have been observed at autopsy in a number of animals. Most of them were instances of long standing syphilitic infection; some animals were in excellent physical condition, while others showed obvious signs of malnutrition for which no cause could be found. In some, the bones contained an abundant red marrow; in others, the marrow was more fatty in character.

It is, of course, well known that similar changes in these bones may be produced through the development of an anemia, and there is at present no reason to regard the processes here shown as the result of a local infection. The circumstances suggest, however, that they may be produced in response to a systemic condition resulting from the syphilitic infection.

*Ulna, Radius, and Tibiofibular.*—Many of the long bones such as the ulna, the radius, and the tibiofibular show little alteration in appearance at any stage of the infection. Usually there was no more than a slight roughening of the surface of the bone with either an increase or a decrease in thickness. These areas also showed a honeycombing of the bone, but the apparent alteration was always less than that shown by microscopic examination.

The tendency was also towards a rapid restoration to normal, and bones examined a few months after the lesion had resolved showed little if any abnormality.

*Metatarsals and Phalanges.*—Gross changes in the metatarsals and the phalanges were more marked. During the active stages of the infection, there were usually surface erosion, necrosis, and disintegration, epiphyseal separation, or fracture. Less often, osseous overgrowth or the laying down of considerable masses of soft, spongy bone occurred, while the lesion was still actively progressing (Fig. 10).

As the infection subsided and the injury to the bone was repaired, various abnormalities were observed. These consisted of changes in the form of the bone, surface roughening, and honeycombing, together with irregular reductions or increases in thickness such as were illustrated for the nasal bones. Many of these changes appeared to be of a semipermanent character. The abnormality diminished with time, but in a few animals which were held for observation, marks of the previous injury were still recognizable several months after the infection had subsided.

*Tarsus*.—Deformities of the tarsus following necrosis, fracture, or epiphyseal separation of the calcaneus were always marked. These conditions invariably led to excessive bone formation. Necrosis and disintegration of the calcaneus were followed by the formation of an irregular granular mass of bone such as that shown in Fig. 32. The union of a fracture or of an epiphyseal separation, on the other hand, was accomplished by the laying down of an excessive amount of callus and the formation of a large mass of spongy bone (Fig. 33). These conditions were also of a more or less permanent character. The absorption of callus and the reestablishment of compact bone took place slowly—more slowly than after ordinary trauma.

#### SUMMARY.

From a study of a series of rabbits inoculated with two old strains of *Treponema pallidum*, it was found that localized infection of bones and tendons was of frequent occurrence and led to the formation of a variety of lesions.

The bones usually involved were those of the face and the feet and legs. Most often the lesions arose from the periosteum but developed also within the bone or marrow cavities and at lines of epiphyseal union.

Grossly, the periosteal lesions were of two types—one being a circumscribed, indurated, and nodular mass and the other a process of a more diffuse character. Histologically, the lesions presented the typical appearance of syphilitic granulomata composed of more or less distinct layers which corresponded roughly with structural divisions of the periosteum. The composition of lesions of membrane and of cartilage bones differed somewhat in this respect, especially in the development of an osteoclastic layer. Invasion of the bone with absorption and necrosis were constant features of periosteal affections and were most marked in the case of the facial bones and the small bones of the feet.

Lesions in the bone and marrow cavities were detected chiefly by radiographs or by the occurrence of bone destruction in the absence of periosteal involvement. They were characterized by a loss of structural detail in the bone, rarefaction, increased fragility, necrosis,

pathological fracture, and epiphyseal separation associated with more or less granulomatous reaction. Histologically, the bone lesions presented essentially the same picture as those of the periosteum, while the lesions which arose from the marrow cavities were composed chiefly of polyblastic infiltrations. In this group of affections, the most important were those which developed at the epiphyses.

The destructive effects produced by all classes of lesions varied from a slight surface erosion or rarefaction to extensive necrosis resulting in the formation of bony defects or in disintegration or fracture of the bone. These conditions differed very decidedly with the particular bones involved.

Of especial importance in this connection was the occurrence of a peculiar form of mass necrosis which at times resulted in the destruction of considerable areas of bone even in parts where the granulomatous type of lesion was comparatively slight. The most characteristic injuries were the saddle-nose deformities and the epiphyseal separation in the small bones of the tarsus and hind feet.

The marks of permanent injury were, on the whole, comparatively slight, but they also differed both with the degree of the original injury and with the bone affected.

Granulomatous lesions of tendons or tendon sheaths were occasionally seen, and in a few instances, lesions of synovial cavities were demonstrated microscopically.

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## EXPLANATION OF PLATES.

The photographs and radiographs represent the objects at their natural size. None of the illustrations has been retouched. Statements of time, unless otherwise indicated, are estimated from the date of inoculation.

## PLATE 52.

FIGS. 1 to 7. Nodular and diffuse periostitis.

FIG. 1. 74 days. Typical periosteal granulomata of the nasal bones, growth tending to be upward rather than inward.

FIG. 2. 123 days. Periosteal granuloma on the shaft of the fifth metatarsal and involving the tendons of the dorsum of the foot. The cut surface shows irregular miliary areas of necrosis.

FIG. 3. 137 days. Periosteal granulomata of the tibiofibular, the fifth metatarsal, and the phalanges. The lesion of the tibiofibular shows a central area of necrosis with softening and a mottling due to congestion and hemorrhage.

FIG. 4. 84 days. Multiple periosteal lesions of the nasal bones.

FIG. 5. The same specimen with the periosteum dissected back. The lesion on the upper part of the nasal bone is of slight extent and shows only a surface erosion of the bone; that over the bridge of the nose is composed chiefly of an osteoclastic growth, and there are complete necrosis and absorption of the bone in this area.

FIG. 6. 2 years and 5 months. Chronic fibrous periostitis of the nasal bones of a diffuse type. The main lesions appear as bulbous masses at the end of the nose.

FIG. 7. The same preparation with the periosteum removed from the left side. There is an irregular thickening of the bone which is most evident in the lower third, and the periosteum was firmly adherent at many points. Note also the irregularity of the end of the nasal bone.

## PLATE 53.

FIGS. 8 and 9. Photomicrographs of periosteal and endosteal granulomata.

FIG. 8. 48 days. Cross-section of nasal bone showing periosteal and perichondrial lesion arising from the inner side of the bone and working its way outward. The external coverings of the bone are normal.  $\times 95$ .

FIG. 9. 89 days. Proximal end of the fifth metatarsal. The photograph shows a combination of lesions, the chief one being a granulomatous nodule in the marrow cavity; there is a distinct widening of this part of the cavity. Lesions are also seen in the bone and on both periosteal surfaces.  $\times 30$ .

## PLATE 54.

FIG. 10. 89 days. An epiphyseal lesion of the distal end of the fifth metatarsal. There has been a separation of the epiphysis which apparently took place in the directions indicated by the arrows. At the time represented in the photograph,

this lesion was in process of repair but there was an active syphilitic granuloma in the epiphysis. A periostitis is also seen to be present and there is an extension of the infection to the synovial membranes lining the joint cavity.  $\times 30$ .

#### PLATE 55.

FIGS. 11 to 14. Radiographs of lesions developing within the bones.

FIG. 11. 119 days. The rarefaction seen at *a* and *b* was due to osteitis of the shaft and proximal end of the metatarsals. The deformity of the tarsus at *c* was due to necrosis of the calcaneus.

FIG. 12. 163 days. Necrosis of the anterior end of the right calcaneus (marked by arrow). There were lesions also at the proximal ends of both lateral metatarsals (marked by arrows).

FIG. 13. 77 days. Pathological fracture of the middle of the calcaneus with effusion into the tissues.

FIG. 14. 73 days. Epiphyseal separation of the right calcaneus. The left calcaneus also shows some degree of abnormality which is indicated chiefly by the thickening of the upper surface of the bone toward its posterior extremity. Normally the shadow of this portion of the bone forms a straight line (*cf.* other radiographs).

#### PLATE 56.

FIGS. 15 to 18. Radiographs showing lesions of the bone.

FIGS. 15 and 16. Successive stages in the development of a lesion of the right calcaneus. Fig. 15 (85 days) shows a peculiar defect in the lower portion of the calcaneus which is marked by an arrow. The left calcaneus is normal. Fig. 16, which was taken 19 days later, shows an outspoken deformity of the right calcaneus. This was due in part to a dropping of the arch from involvement of the ligaments and in part to necrosis of the calcaneus, the talus becoming embedded in its substance. Note the relative position of the talus in Figs. 15 and 16.

FIGS. 17 and 18. Stages in the development of an epiphyseal separation. In Fig. 17 (77 days) there is a broad band of rarefaction at the epiphyseal line of the right calcaneus (marked by arrow) and a suggestion of a separation is seen at the top of this line. The posterior end of the left calcaneus also shows a narrowing of the neck which is abnormal. Fig. 18, taken 8 days later, shows a separation of the epiphysis of the right calcaneus which followed the line indicated in Fig. 17.

#### PLATE 57.

FIGS. 19 and 20. Photomicrographs showing characteristic periosteal lesions of membrane and cartilage bone.

FIG. 19. 60 days. Periostitis of the nasal bone showing an outer cellular layer and an inner osteoclastic layer. The cellular layer is composed chiefly of fibroblasts with a moderate degree of polyblastic infiltration. It is also quite vascular. The osteoclastic layer is composed of fibroblasts and polyblasts together with a large number of osteoclastic cells.  $\times 95$ .

FIG. 20. 77 days. Periostitis of the metatarsal. The lesion shows three distinct layers which present much the same general appearance. The two outer layers show a considerable degree of polyblastic infiltration; they are more vascular than the inner layer and the vessels show well marked endarteritis. The osteoclastic layer is but slightly developed; it is largely fibrous in character and contains very few osteoclasts.  $\times 95$ .

#### PLATE 58.

FIGS. 21 and 22. Photomicrographs, taken from the nasal bone, showing different types of bone destruction.

FIG. 21. 60 days. Necrosis and absorption of bone accomplished chiefly by surface action. Note the wave lines in the bone, the preservation of architecture, and the survival of bone corpuscles.  $\times 135$ .

FIG. 22. 100 days. Mass necrosis with rapid disintegration of bone—a type of lesion frequently observed in cases of marked bone destruction as it occurs in an acutely progressive process.  $\times 135$ .

#### PLATE 59.

FIGS. 23 to 26. Photographs of dried preparations showing the maximum bone injury produced by various types of lesions.

FIG 23. 121 days. Surface erosion of the nasal bones produced by a large periosteal granuloma, the growth of which was mostly in the outer layers.

FIG. 24. 118 days. Necrosis of the nasal bones with the production of saddle-nose deformity due to an invasive periosteal lesion.

FIG. 25. The same specimen viewed from the side.

FIG. 26. 124 days. A defect of the right nasal bone produced by a lesion arising from the inner side of the bone. There was only a suggestion of periosteal thickening in this area.

#### PLATE 60.

FIGS. 27 to 33. Bone lesions and the deformities produced in the process of repair.

FIG. 27. 123 days. A widespread necrosis of the nasal bones resulting from a diffuse periostitis which produced only a slight palpable thickening over the surface of the bone.

FIG. 28. 137 days. Similar lesion viewed from the side. Note especially the destruction of the margin of the bone, the involvement of the lateral splints, and extension of the lesion to the premaxilla.

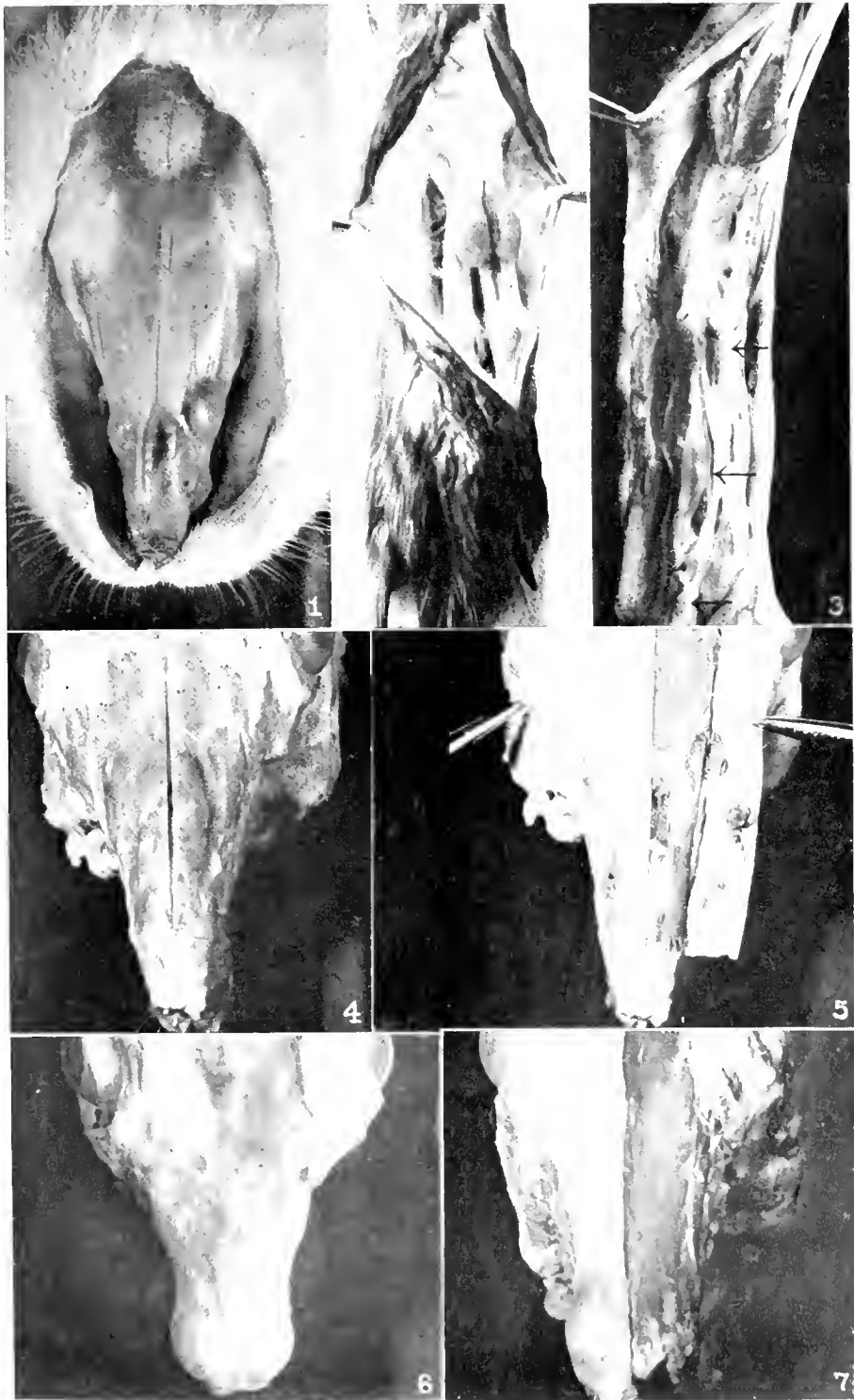
FIG. 29. 121 days. An imperfect regeneration of the nasal bones following almost complete necrosis of the areas involved. In the upper portion of the right nasal, the bone is very irregular, while below it is comparatively smooth but considerably thickened, and a defect is still present in this part of the bone.

FIG. 30. 130 days. An osteophytic node at the point of union of the frontal process of the premaxilla and the maxillary process of the frontal bone (nasal splints), illustrating a tendency of syphilitic lesions to become localized at points of bony union.

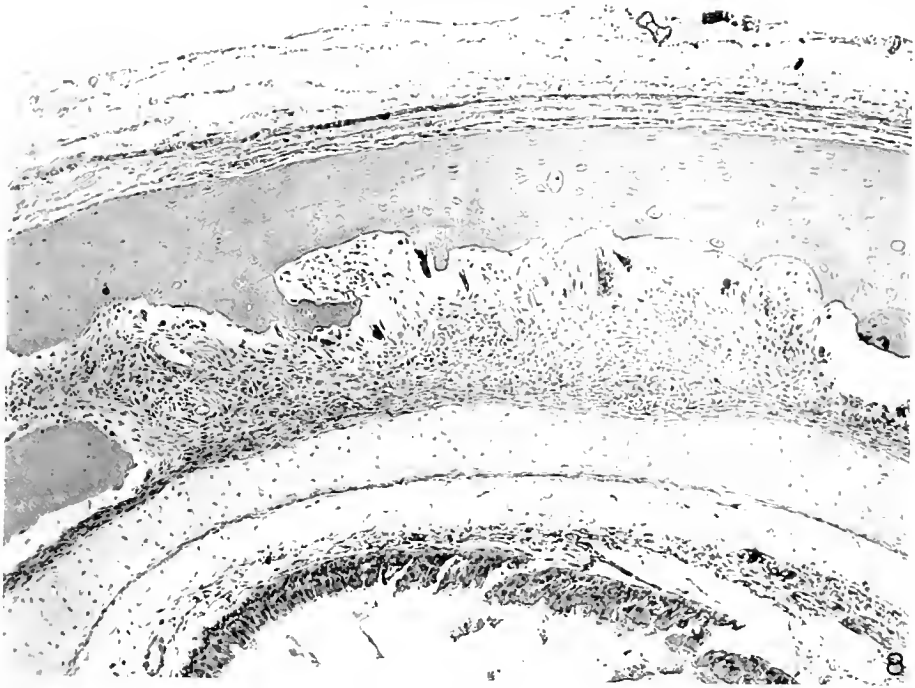
FIG. 31. 342 days. A thickened spongy condition of the parietal and occipital bones occurring late in the course of a generalized infection. The exact cause of the condition is not known.

FIG. 32. 126 days. A deformity of the tarsus resulting from necrosis of the calcaneus. This bone is considerably thickened and extremely irregular.

FIG. 33. 126 days. A deformity produced by an epiphyseal separation of the calcaneus. The original lesion healed with the formation of a large mass of callus about the posterior end of the calcaneus and the separated epiphysis.

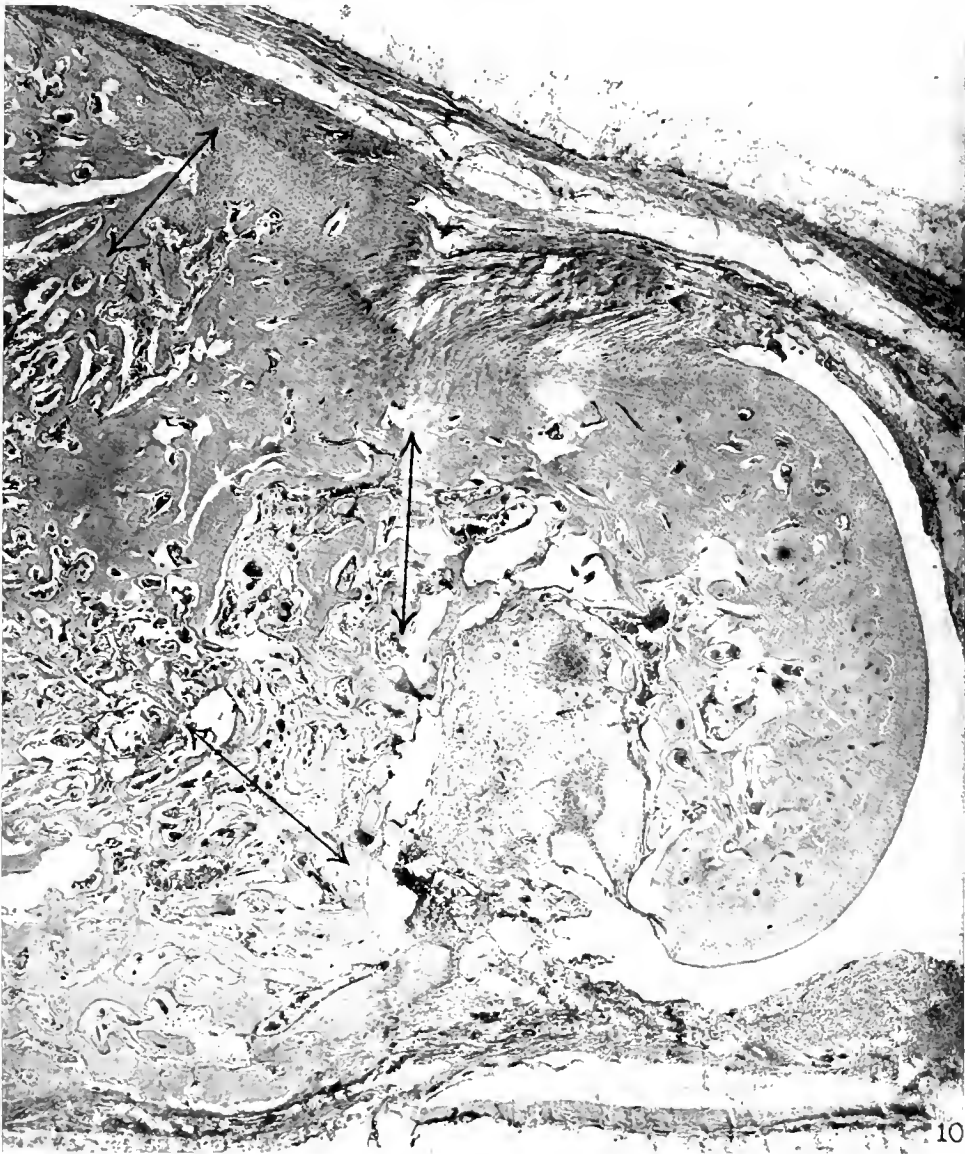






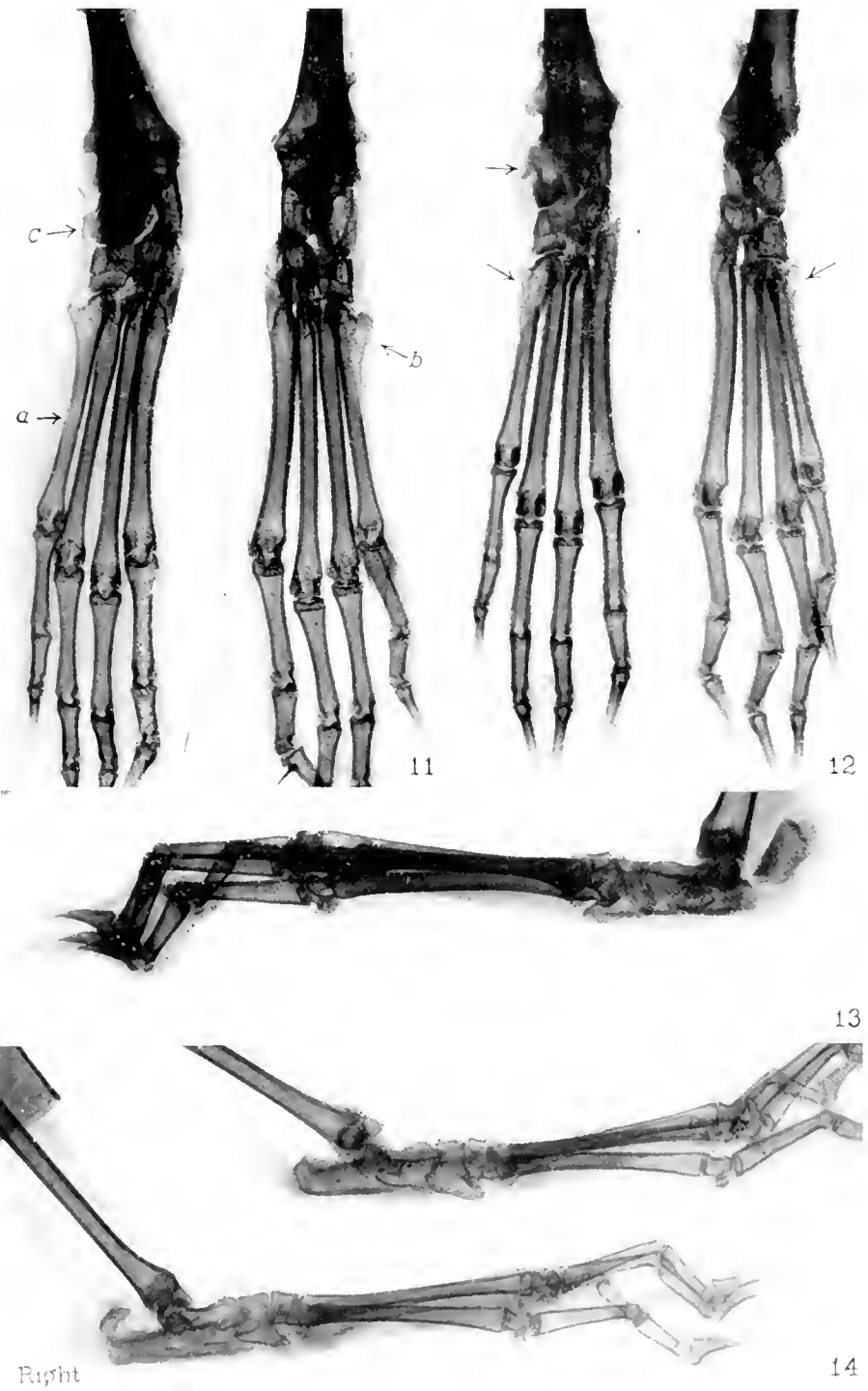






(Brown, Pearce, and Witherbee. Experimental syphilis in the rabbit. VI.)

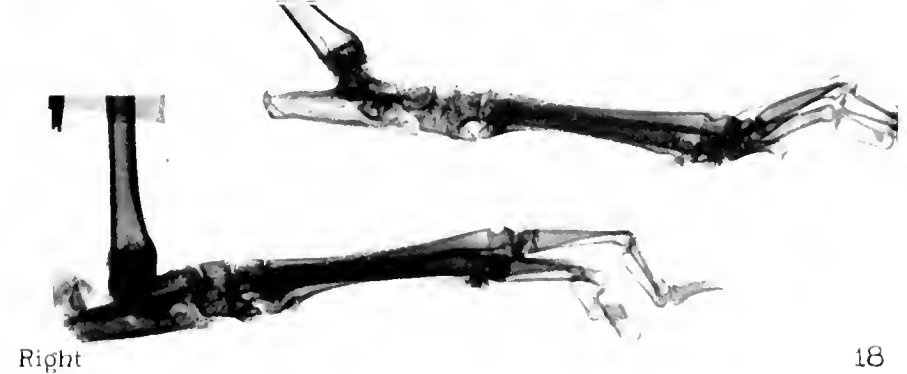
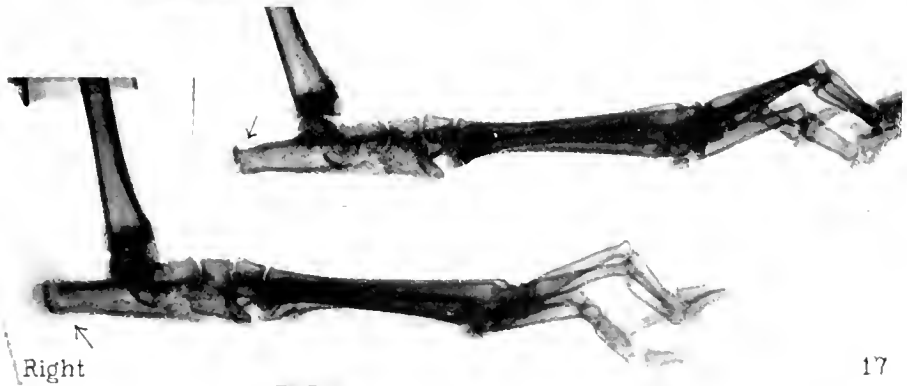
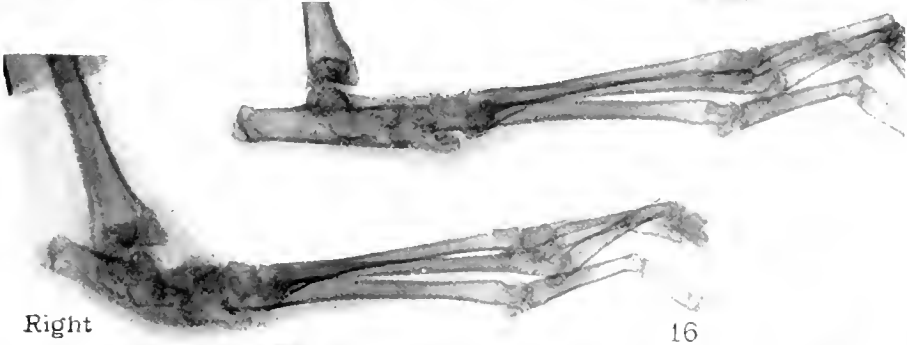
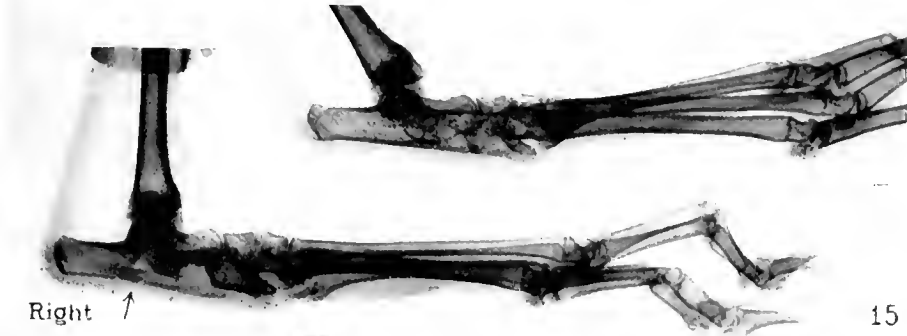




(Brown, Pearce, and Witherbee. Experimental syphilis in the rabbit. VI.)

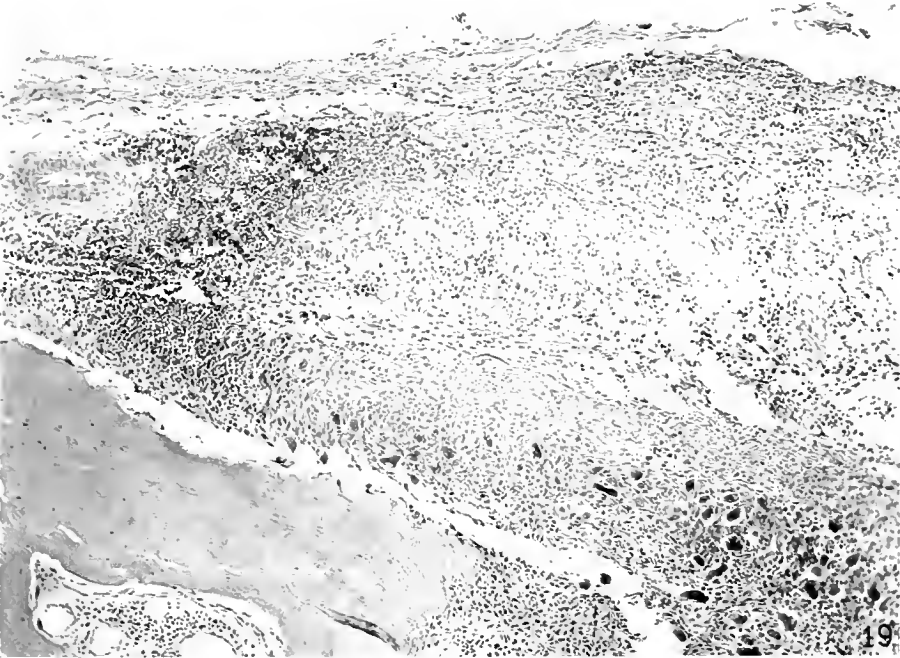


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(Brown, Porter, and Witherbee: Experimental syphilis in the rabbit. VI.)





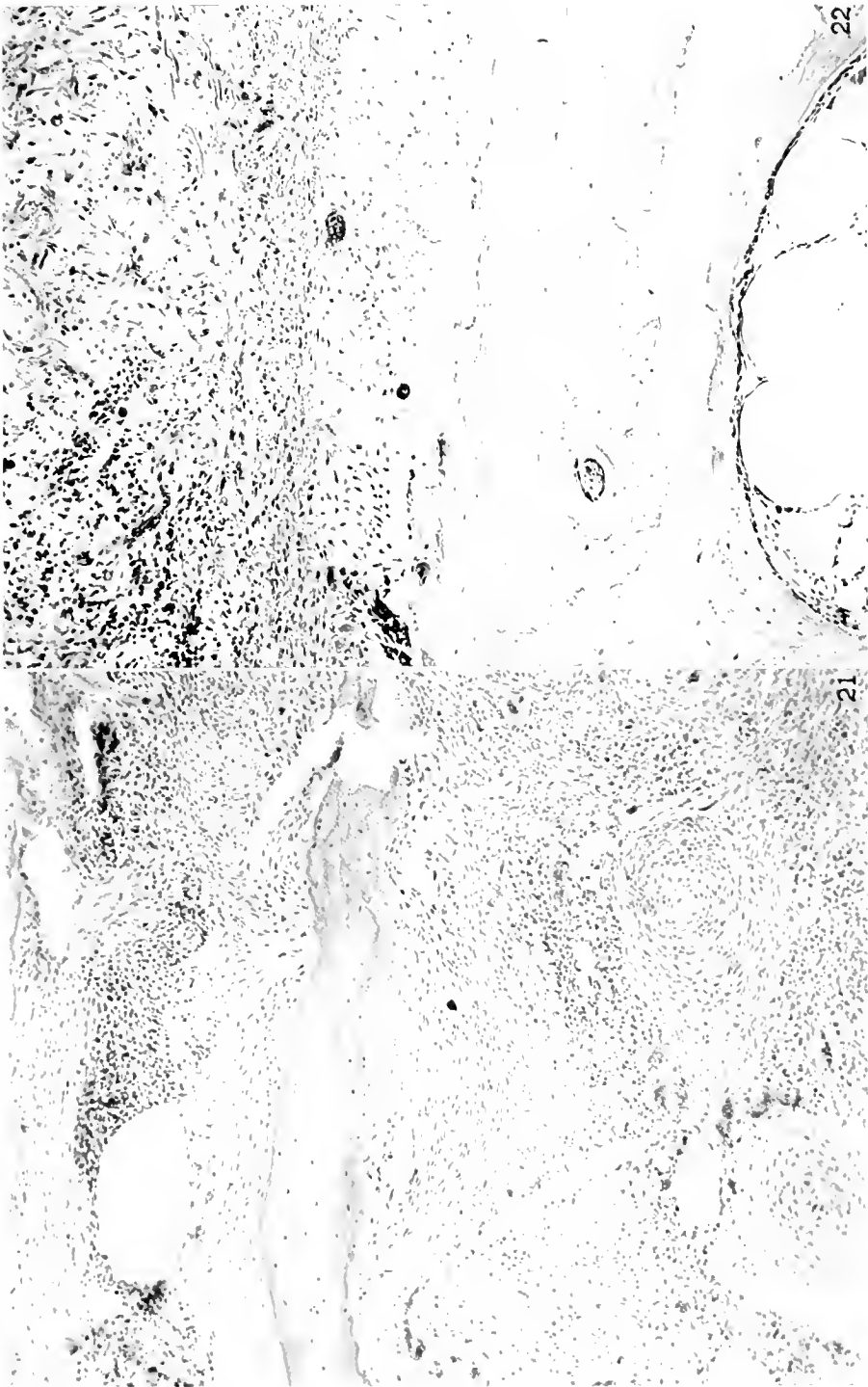
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(Brown, Pearce, and Witherby: Experimental syphilis in the rabbit. VI.)



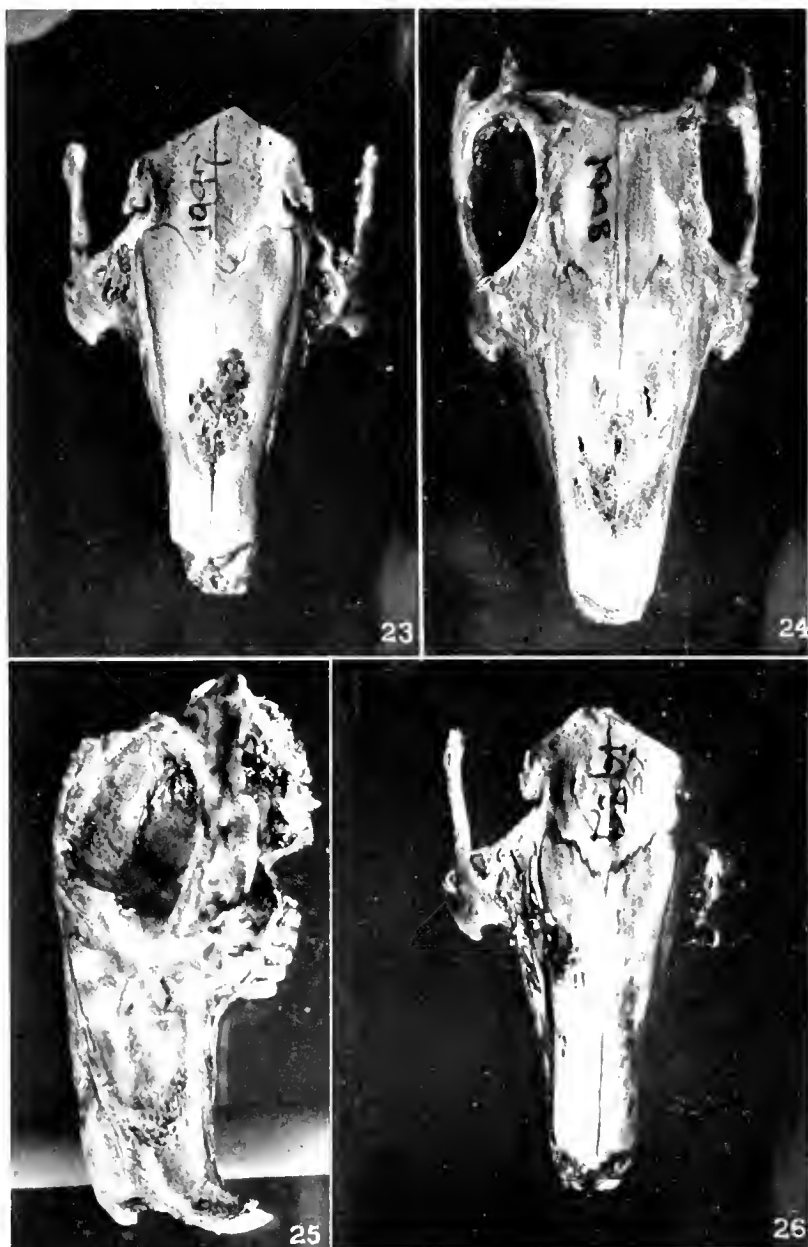


FIG. 1. Pearce, and Witherby. Experimental syphilis in the rabbit. VI



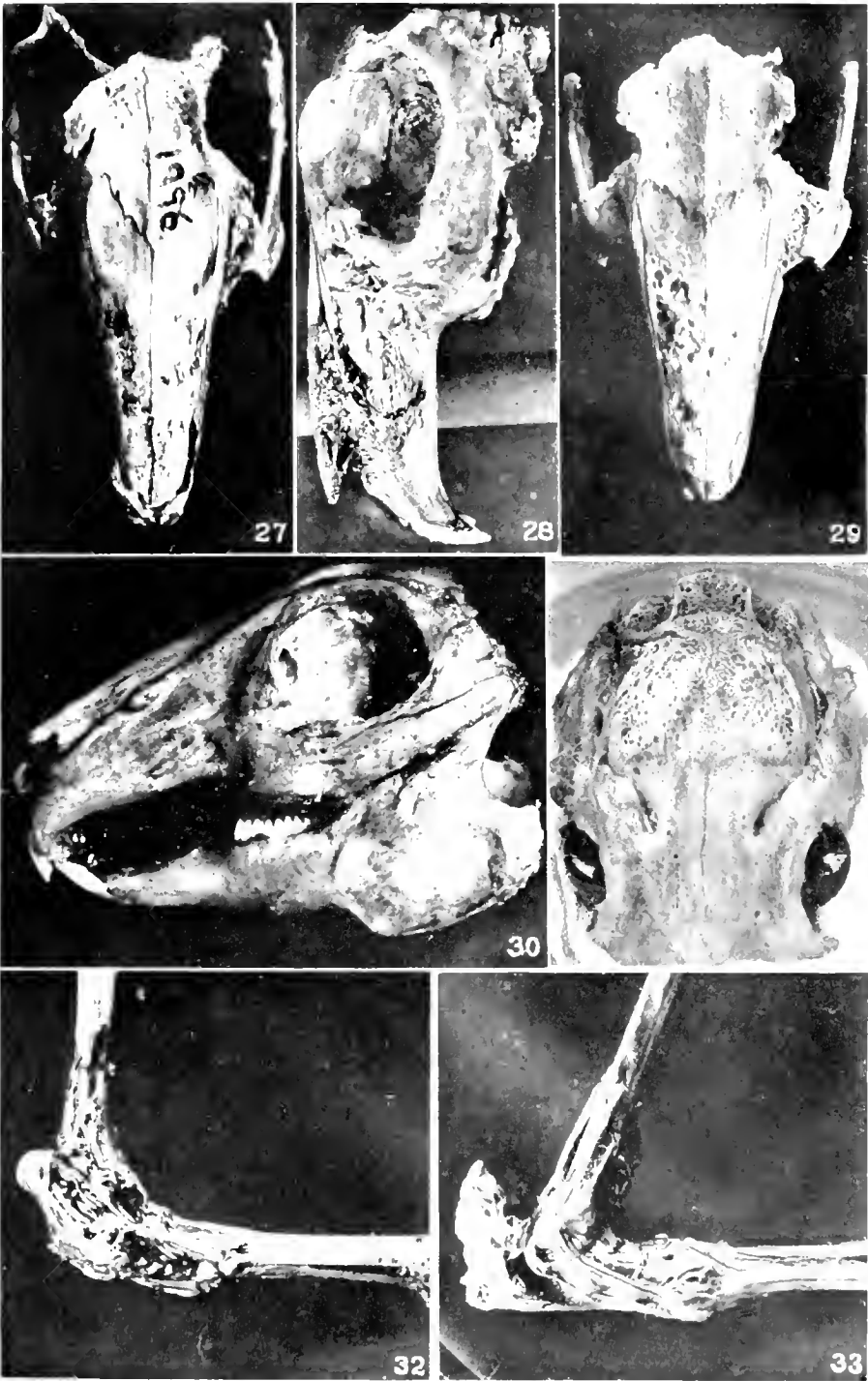


FIGURE 27. 28. 29. 30. 31. 32. 33. WITH THE JOURNAL OF EXPERIMENTAL MEDICINE



## EXPERIMENTAL SYPHILIS IN THE RABBIT.

### VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

#### PART 2. CLINICAL ASPECTS OF SYPHILIS OF THE SKELETAL SYSTEM. AFFECTIONS OF THE FACIAL AND CRANIAL BONES AND THE BONES OF THE FOREARM.

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PLATES 61 TO 66.

(Received for publication, January 31, 1921.)

Syphilitic affections of the skeletal system were described in the preceding paper (1) from the standpoint of the pathological process, and it is the purpose of this paper to correlate these changes with clinical manifestations of the disease. This phase of experimental syphilis is of unusual importance on account of the widespread and frequently obscure nature of the infection. The subject will be dealt with, therefore, in somewhat greater detail than other groups of affections with the belief that the facts presented have an important bearing upon latent or obscure infections in the human subject and especially upon congenital forms of the disease.

The conditions to be described are numerous and it will be necessary to divide the material into two papers, the first of which will deal with affections of the facial and cranial bones and the bones of the forearm. The remaining subjects will be considered in the second paper.

#### *Parts Involved and Character of the Affection.*

The most obvious affections of the skeletal system were those of the face and of the feet and legs, but lesions were also found in many other parts of the body, such as the cranial bones, the cervical and

caudal vertebræ, the ribs, and the sternum. The proportion of focal infections which gave rise to clinical signs of disease cannot be estimated, but it seems probable that many obscure infections occurred which did not give rise to any obvious abnormality.

Bone infection was recognized in general by the development of visible or palpable enlargements over the surface of the bones, or, in the absence of these, by rarefaction and the loss of structural detail as indicated by the radiograph, and eventually by necrosis and disintegration of the bone. The skin over the lesion was freely movable, while the nodular mass was firmly attached to the underlying bone. In rare instances, the skin might become adherent, but it is worthy of note that necrosis and ulceration of the skin were never observed in connection with lesions of either tendons or bones, with the exception of the phalanges. The diagnosis of these conditions as syphilitic affections was readily made by the demonstration of spirochetes in the lesions.

These were the general characteristics presented by all classes of bone infections, but the details of the processes as they occurred in different parts of the body were subject to considerable variation.

#### *Facial and Cranial Bones and Cartilages.*

Judged upon the basis of the occurrence of lesions which were sufficiently pronounced to be detected by ordinary methods of examination, the facial bones were more often the seat of syphilitic affections than those of any other part of the body, with the possible exception of the hind feet and legs. The bones usually involved were the nasals, the two lateral splints composed of the frontal process of the premaxilla and the maxillary process of the frontal, the turbinates, and to a lesser extent the maxillæ, the premaxilla, and the frontal eminences. These bones lie immediately around the nasomaxillary fossa, and, with few exceptions, the lesions of the facial area were confined to the region of this fossa and the bridge and sides of the nose. The most frequent locations were the bridge of the nose, the end of the nose, and the bony ridge extending from the naso-orbital angle along the sides of the nose, while young animals in particular showed a marked tendency to localization at the point of union of the frontal and maxillary processes.



The appearance presented by animals with well marked facial lesions of various types is shown in Figs. 1 to 7, 11, and 15. Fig. 1 represents a very common type of deformity due to a sharply circumscribed periosteal lesion on the bridge of the nose. Fig. 2 is a more pronounced condition of a similar character which involved the lower two-thirds of the nasal bones and the lateral splints and extended well down on the premaxilla.<sup>1</sup> The profile of the animal shows a gradual curve extending from just below the level of the eyes to the tip of the nose, the deformity being less abrupt than that in the preceding case, and hence not so noticeable at first.

A less common affection is that illustrated in Fig. 3. This was a multinodular lesion of the lower portion of the nasal bones and cartilages and was the most pronounced case of its kind which occurred in our series of animals, measuring 1.85 by 1.7 by 1.2 cm.

Another unusual type which was associated with a marked alteration of the profile is that shown in Fig. 4. The prominence seen above the eye of this animal was due to a large periosteal granuloma involving the supraorbital portion of the frontal bone. This process was bilateral.

The visible deformity in all these animals was quite apparent, but there were many instances in which little or no visible alteration in contour could be detected either on account of the size and form of the lesion or its location. This was especially true when the sides of the nose and the region of the nasomaxillary fossa were involved. Photographic reproduction of even large lesions in these locations was found to be extremely difficult. This class of affections is illustrated by two animals (Figs. 5 and 6) both of which represent very common types of facial involvement.

The lesions of the animal in Fig. 5 consisted of a multinodular mass which extended downward along the bony ridge at the sides of the nose from the region of the naso-orbital angle and then across the bridge of the nose. They were quite large and yet very little of the lateral deformity could be seen until the hair was clipped, disclosing the marked increase in breadth or fullness of the midnasal region which is fairly well shown in the photograph.

In Fig. 6, there is a large, flat, and slightly oval lesion covering the upper half of the right nasal bone and extending well back over the nasomaxillary fossa. It measured more than 0.5 cm. at its center (determined after resection), and yet no deformity of the face was apparent until the hair was removed.

In like manner, the symmetrical deformity produced by many bilateral lesions was even less evident than that of unilateral affections,

<sup>1</sup> See Brown, Pearce, and Witherbee (1), Fig. 28.

especially in the naso-orbital angles or along the upper portions of the nasal splints, which are frequently involved. The appearance presented in the majority of these cases was merely that of a fullness between the eyes or of a slight prominence in the naso-orbital curve.

Facial abnormalities were more apparent with periosteal lesions arising from the outer surface of the bones than with other forms of bone involvement, unless the lesions extended to the outer surface, in which case they frequently assumed an appearance similar to the conditions described.

The extent of the process and the damage produced by infections of the facial bones bore no particular relation to the size of the external growth. These changes could be gauged by palpation, by sounding the lesion with a pointed instrument, or by radiographs. The information to be derived from the use of the latter method of investigation may be illustrated by a series of animals in which the appearance of the lesion, the radiograph, and the bone specimen are compared (Figs. 7 to 16).

The first animal in this series had a marked diffuse periostitis of the nasal bones which produced a typical Roman nose deformity (Fig. 7). By palpation, it was found that the entire lower portion of the bone gave beneath the finger, and a radiograph taken at this time (Fig. 8) showed complete destruction of the mid-area of the nasal bones together with some clouding and loss of architecture in adjacent parts of the bone. After about 2 weeks, the lesion began to subside and regeneration of the bone could be detected. At the end of 6 weeks, a second radiograph (Fig. 9) showed a marked but irregular thickening of the nasal bones and an absence of the usual architecture. There was also a bony mass extending below the level of the nasal bones. The actual condition existing is shown by Fig. 10 which is a photograph of the dried skull.

A second, more significant case is illustrated in Figs. 11 to 14. This was an instance of diffuse periostitis which produced very little facial deformity. A slight thickening could be made out over the bridge and lower portion of the nose and a defect could be detected along the sides of the nasal bones near the end of the nose. The radiograph of these lesions showed very plainly that there was some bone destruction as indicated by the ragged appearance and the thinning out of the under side of the shadow near the end of the nose (*cf.* Fig. 13 which shows the normal appearance). The fine lines of shadow produced by the turbinates were also blurred, and there were clouding and loss of architecture throughout most of the nasal bone. When these findings were compared with the autopsy specimen (Fig. 14), it was found that the destruction was somewhat greater than might have been imagined from the radiograph.

The late effects following repair in a similar case of diffuse periostitis are shown in Figs. 15 and 16. In this animal, there was a permanent enlargement of the end of the nose (Fig. 15) due in part to a fibrous thickening of the periosteum and in part to a thickening of the nasal bones as shown in Fig. 16. The condition had existed for upwards of 2 years.

Infections arising from the interior of the nose were characterized clinically by one or more of three conditions: the presence of a nasal discharge containing spirochetes, necrosis of the outer covering of bone, and finally, the development of external granulomatous lesions. Few of these infections have been recognized clinically and our knowledge of them is mostly that of autopsy findings. Involvement of both the nasals and the turbinates has been demonstrated in this way, but it will be seen at once that in the absence of any external lesion, there is no simple method of recognizing affections of this class or of differentiating them from affections of the mucous membranes. It is not improbable, therefore, that many internal lesions may escape detection and that many instances of nasal discharges containing spirochetes and of obstructive phenomena of the nasal passages are referable to bone infection rather than to primary infection of the mucous membranes. This supposition is supported by abundant pathological evidence.

The probability of the occurrence of lesions other than those described must not be overlooked. Several instances of peculiar thickening of the frontal and parietal bones of rabbits with generalized syphilis have been noted, and there was one animal with a marked mandibular affection. These lesions were all inactive, and there was nothing present which would enable one to determine their cause with certainty. However, a case of necrosis of an occipital condyle was observed, in which the infection was still active. The circumstances in this case will be given later (2).<sup>2</sup>

The points of especial interest in connection with syphilis of the facial bones are the frequency, the location, and the destructiveness of the lesions. It is also important to note the close analogy which exists between certain of the nasal and supraorbital affections of the experimental animal and those of man, especially in the congenital form of the disease.

<sup>2</sup> Brown, Pearce, and Witherbee (2), p. 531.

*Forearms.*

Bone affections of the anterior extremities of the rabbit were, with one exception, represented by a periostitis involving the distal ends of the ulna and radius. The majority were situated on the extensor surfaces at or near the epiphyseal line, with an occasional lesion at a slightly higher level.

This type was of comparatively common occurrence. As a rule, the lesions were rather small and with the fur intact produced little or no alteration in the appearance of the affected part but were easily detected by palpation. Normally, the extensor surface of the forearm is either perfectly smooth or is marked by small cross ridges and angular projections at the epiphyseal lines and the heads of the radius and ulna respectively. When lesions develop at these points, oval or rounded nodules are formed which are very readily felt on examination and can be seen after removal of the hair.

A typical case of bilateral involvement of both ulna and radius is shown in Fig. 17 (*cf.* Fig. 18 in which the left forearm is normal). Three of the four nodes present were distinctly rounded, while that on the right radius was more oval and covered a greater area of the bone.

A second type of affection which was frequently encountered is that seen in Fig. 18. In this instance, there was only one lesion, which formed a rather large oval mass on the right ulna just above the carpus.

In exceptional instances, the lesions of these bones were much more marked than those described and produced very striking deformities, as in Figs. 19 and 20. This, again, was a bilateral affection of both the ulna and the radius. As may be seen from Fig. 20, the lesion of the ulna was very sharply demarcated and raised abruptly from the surface of the bone, while that of the radius was more diffuse and resulted in extensive necrosis of the distal end of the bone.

A second case of an even more marked character is illustrated in Figs. 21 and 22. This was a large oval lesion which arose from the ulna but extended to the radius by way of the interosseous membrane. It was one of the few instances in which definite necrosis of the bones of the forearm could be made out clinically. As shown in the accompanying radiograph (Fig. 22), there was necrosis of the lower end of the ulna as well as the contiguous margins of both the ulna and the radius. The lesion was of very rapid growth, reaching the stage shown within 9 days after it first appeared. The tissues surrounding it were of a violet-red color, and there was a pronounced edema of both the skin and subcutaneous tissues.

In addition to the affections described, mention may be made of an enlargement of the carpus, which was followed by a permanent deformity occurring among the first animals of our series, but the methods then in use were not sufficiently developed to enable us to determine the nature of this condition. From the circumstances, it seems probable, however, that this might have been due to syphilitic infection. No other case of the kind has come under our observation.

The examples of periostitis of the ulna and radius which have been cited were cases in which there was not more than one lesion on each bone. Multiple lesions of one or both bones were occasionally seen, but they were usually small. In many instances, only one forearm was affected, but more often the involvement was bilateral and symmetrical.

It was a notable feature of this group of lesions that they rarely led to extensive necrosis. Grossly, the surface of the bone was eroded or roughened, and obliteration or widening of the epiphyseal line could be demonstrated by use of x-rays. Similarly, when the lesion healed, the bone might show a slight roughening or increase in thickness with a few tiny nodes (see lateral margin of the ulna in Fig. 22), but rarely was there any considerable deviation from the normal. Microscopic examination showed, however, that the syphilitic process frequently extended through the entire thickness of the bone and that the effect produced was in general much greater than gross appearances would indicate.

Other aspects of the subject of syphilis of the skeletal system will be presented in Part 3 of this paper (2).

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## EXPLANATION OF PLATES.

All illustrations are reproductions of photographs or radiographs which have not been retouched and represent objects at their natural size. Statements of time are estimated from the date of inoculation unless otherwise indicated.

FIGS. 1 to 6. Appearance presented by animals with syphilitic affections of the facial and cranial bones.

## PLATE 61.

FIG. 1. 146 days. Nodular periostitis, bridge of the nose.

FIG. 2. 105 days. Marked diffuse periostitis of the lower two-thirds of the nasal bones.<sup>1</sup>

FIG. 3. 159 days. Marked nodular periostitis and perichondritis of the nasal bones and cartilages.

FIG. 4. 71 days. Marked periostitis of the supraorbital region.

## PLATE 62.

FIG. 5. 110 days. Multinodular periostitis of the nasomaxillary fossa, the sides and bridge of the nose. The lesions were very pronounced.

FIG. 6. 92 days. Periostitis of the right nasal bone and splint. The lesion formed a broad flattened mass which covered the upper half of the bone and adjacent portions of the fossa.

FIGS. 7 to 16. A comparative study of clinical and pathological conditions in syphilitic affections of the nasal region.

## PLATE 63.

FIGS. 7 to 10. Marked diffuse periostitis of the nasal bones and splints with necrosis and regeneration.

FIG. 7. 68 days. The deformity of the nose. The lesion was centered over the bridge of the nose and extended from just below the level of the eyes to the tip of the nose.

FIG. 8. 68 days. Radiograph showing complete necrosis of the bone at the center of the process. The turbinates are also involved.

FIG. 9. 6 weeks later. Regeneration of bone. The nasal bones are considerably thickened, irregular, and devoid of architecture. There is also an irregular osteoid mass beneath the nasal plates.

FIG. 10. 121 days. Lateral view of the skull showing the regenerated bones.<sup>3</sup>

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<sup>3</sup> For frontal view see Brown, Pearce, and Witherbee (1), Fig. 29.

## PLATE 64.

FIGS. 11, 12, and 14. Diffuse periostitis which produced very little alteration in the facial contour.

FIG. 11. 102 days. A slight prominence towards the end of the nose was the only visible abnormality. Palpation showed necrosis of the margins of the nasal bones.

FIG. 12. 116 days. Radiograph showing clouding of the nasal bones and erosion of the under side near the end of the nose. There are also a marked clouding and loss of finer shadow details throughout the nasal chambers and parts of the maxilla and premaxilla, indicating an extensive involvement of these parts.

FIG. 13. Normal radiograph for comparison.

FIG. 14. 123 days. Skull of the animal in Figs. 11 and 12.<sup>4</sup>

## PLATE 65.

FIGS. 15 and 16. 2 years and 2½ months. Chronic fibrous periostitis with bulbous thickening of the end of the nose—a permanent condition. The enlargement of the nose was due in part to fibrous tissue and in part to thickening of the bone. In the radiograph (Fig. 16) note the change in shape and prolongation of the nasal bones, eburnation, and increase in osteoid tissue on the under side of the nasal bones and throughout the nasal region.<sup>5</sup>

FIGS. 17 to 22. Syphilitic affections of the radius and ulna.

FIG. 17. 61 days. A typical bilateral periostitis of the radius and ulna.

FIG. 18. 92 days. A typical case of unilateral periostitis of the ulna (right) with extension to the interosseous membrane and the adjacent margin of the radius. The left forearm is normal.

## PLATE 66.

FIG. 19. 76 days. An unusually marked affection of both the ulna and radius. The condition was bilateral.

FIG. 20. 77 days. Autopsy specimen of the same lesions. That of the ulna is seen to be a sharply demarcated periosteal granuloma, while that of the radius is an osteitis of the head of the bone.

FIG. 21. 60 days. Marked periostitis and osteitis of the distal ends of the ulna and radius.

FIG. 22. 61 days. Radiograph of the same lesions. Note the irregularities and rarefaction of the lower ends of the bone and the fusiform enlargement of the radius.

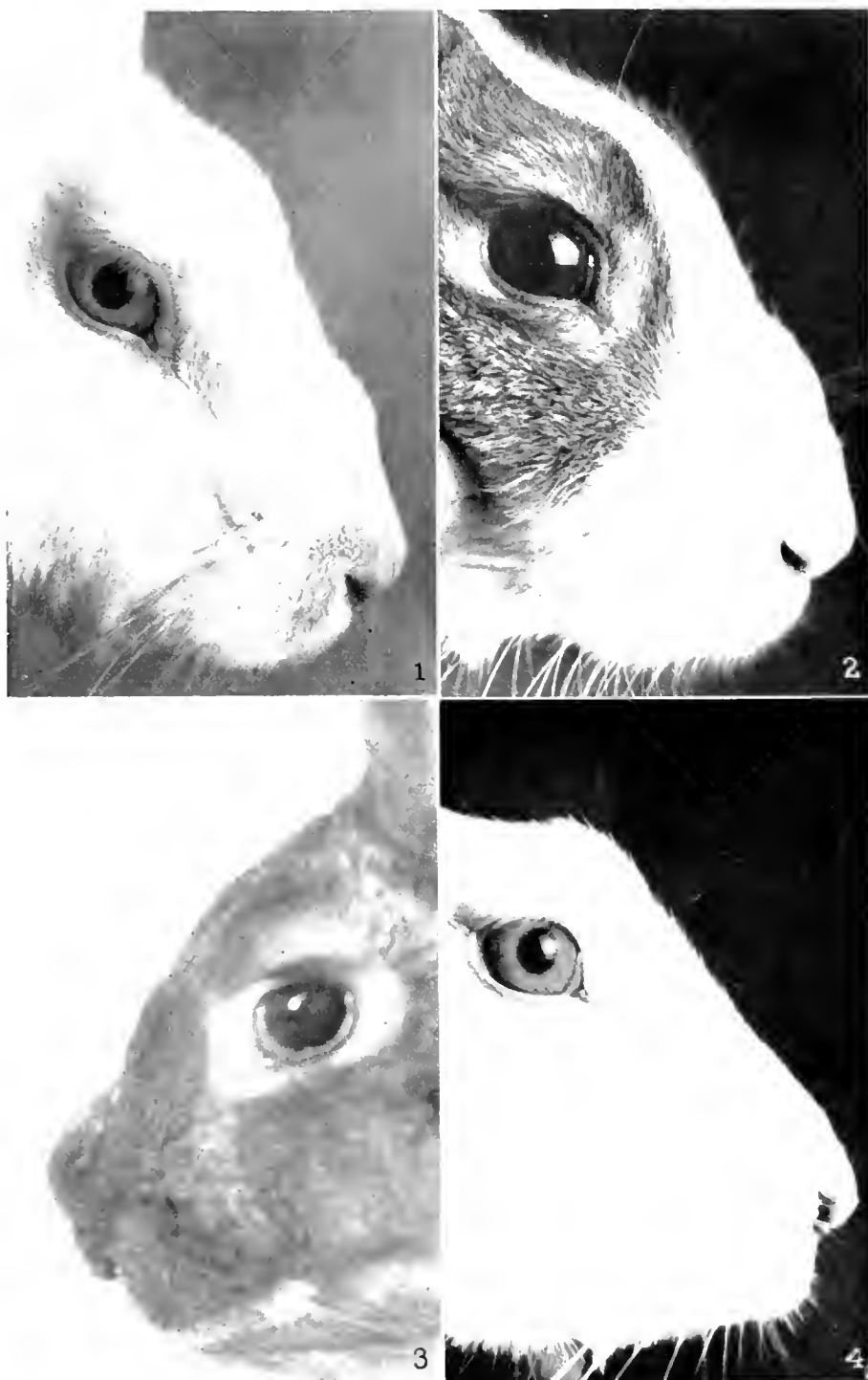
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<sup>4</sup> For frontal view see Brown, Pearce, and Witherbee (1), Fig. 27.

<sup>5</sup> For skull see Brown, Pearce, and Witherbee (1), Figs. 6 and 7.







Brown, Pearce, and Witheluck. Experimental yphid on the rabbit. VI





Brown, Pearce, and Witherby. Experimental syphilis in the rabbit. VI



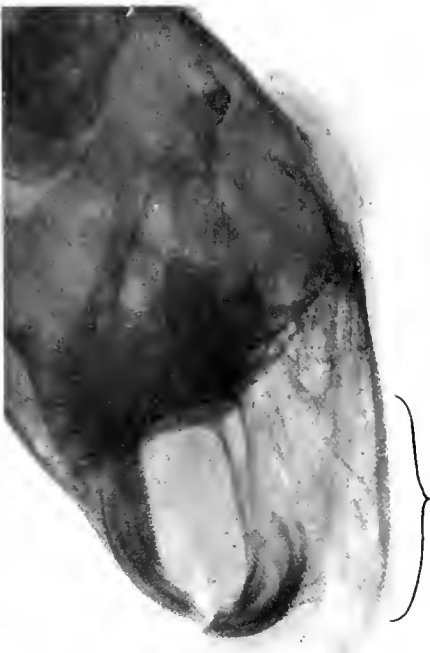


Brown, Pearce, and Witherbee: Experimental syphilis in the rabbit. AL.

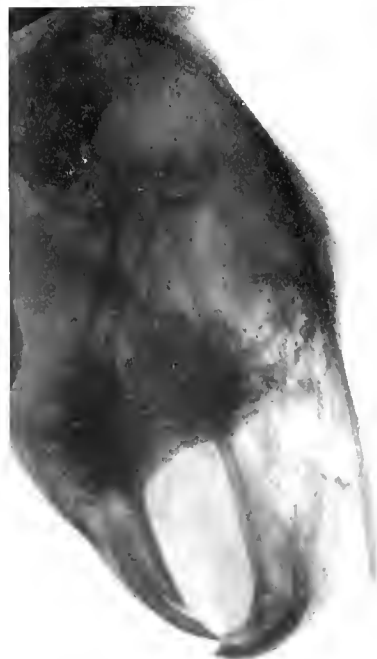




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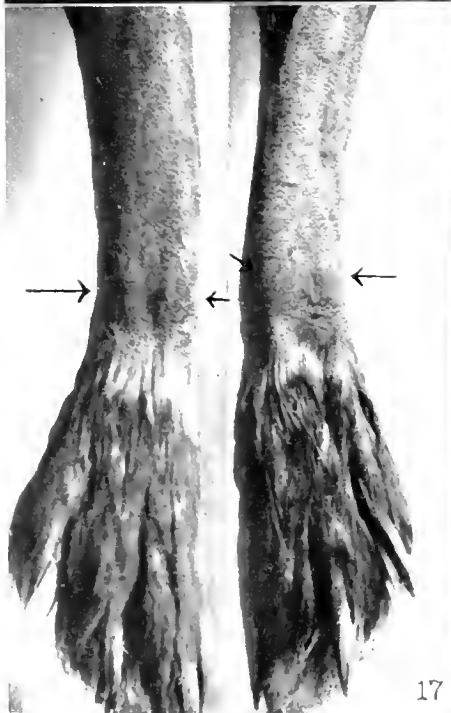
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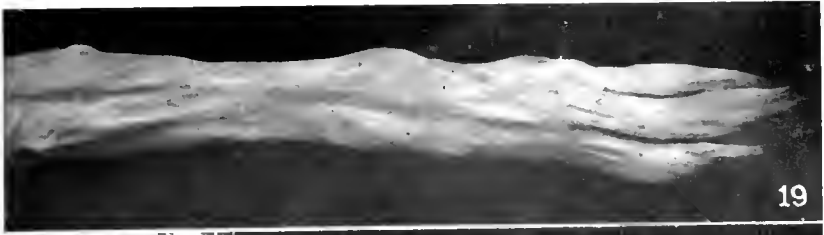






Brown, Pearce, and Witherbee. Experimental syphilis in the rabbit. VI.







## EXPERIMENTAL SYPHILIS IN THE RABBIT.

### VI. AFFECTIONS OF BONE, CARTILAGE, TENDONS, AND SYNOVIAL MEMBRANES.

#### PART 3. SYPHILIS OF THE POSTERIOR EXTREMITIES WITH OTHER AFFECTIONS OF A MISCELLANEOUS TYPE.

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PLATES 67 TO 72.

(Received for publication, January 31, 1921.)

The phases of syphilis of the skeletal and supporting systems to be considered in this paper are the affections of the hind feet and legs, and of the vertebral column, joint cavities, and tendons with the clinical history of this form of experimental syphilis.

#### *Hind Feet and Legs.*

In our first series of animals, there were fewer instances of involvement of the bones of the hind feet and legs than of the forearm, which was the reverse of the condition noted with the cutaneous lesions. Among the more recent cases of generalized syphilis, however, the incidence of bone lesions of the posterior extremities was markedly increased, and the lesions themselves were much more pronounced. The bones involved were the lower end of the tibiofibular, the tarsals, the metatarsals, and the phalanges. The affections included both periosteal and endosteal processes with a considerable number of cases resulting in epiphyseal separation or pathological fracture.

*Tibiofibular.*—The most easily recognizable lesions of the lower extremities were those of the tibiofibular, all of which were periosteal in origin and with a single exception occupied essentially the same position; namely, the anterolateral surface of the lower end of the bone, or what might be termed the external malleolus. This position

corresponds to the line of union between the tibia and the fibula and normally is marked by a small depression surrounded by a series of angular points or ridges. The development of a periosteal lesion in this location caused a filling in of the depression and rounding off of the bony prominences.

Affections of this type were quite common. Some of the lesions were of a comparatively small size, while others reached a centimeter or more in diameter and produced a very characteristic deformity, as may be seen by reference to Figs. 1 to 3.

In this animal, there was a bilateral involvement of the bones, the lesions of the two sides being of approximately the same size and character. The right leg (Fig. 1) illustrates the appearance presented with the fur intact, while the left, with the hair removed, shows more accurately the location of the lesion and the actual deformity produced. The autopsy preparation in Fig. 2 shows the appearance with the skin reflected.

None of the affections of this group appeared to produce any considerable gross alteration in the bone. The changes noted were analogous to those of the ulna and radius. Fig. 3, which is a radiograph of the lesions in Fig. 1, shows the condition usually found. On the right, there is a very definite thinning of the outer table and some irregularity in the surface of the bone. The surface of the left tibia is less affected and shows no more than a suggestion of a roughening, but there appears to be some rarefaction in the lower end of the bone, and the most marked alterations were probably in the heads of the bones. This radiograph may also be used as a means of orientation for the lesions and the parts of the bone affected.

Minor variations in the location of the lesions were not uncommon, but in only one instance was the position materially different from that described. In addition to the usual involvement of the external malleolus, this animal developed a third lesion which took the form of an annular periostitis surrounding the shaft of the right tibiofibular over an area about 1 cm. in breadth and at a level of 2 to 3 cm. above the distal end of the bone.

*Metatarsals.*—While affection of the metatarsals was of frequent occurrence, the lesions were, with few exceptions, confined to the so called fifth or lateral metatarsal; the other bones involved were the first and the fourth. This group of affections presented a variety of conditions consisting chiefly of periosteal lesions but including also affections of the bone and a considerable number of cases of epiphyseal

separation. While some were small and rather difficult to detect, except by the most careful palpation, the usual affection was quite obvious to one familiar with the topography of this part of the foot.

The fifth metatarsal of the rabbit is much the same as that of man. The features of especial importance are the presence of a sharp bony prominence, or hook, formed by the tuberosity at the proximal end of the bone and the narrow tendinous ridge which extends along the lateral margin of the foot throughout the length of the bone. These features of the normal foot are shown in Fig. 4.

The tuberosity, or proximal end, of the metatarsal was perhaps most frequently affected. Irrespective of its origin, the condition usually produced was of the nature of a firm swelling which centered about the head of the bone and extended some distance along the adjacent portion of the shaft. The picture presented by such affections was that shown in Fig. 5. The bone injury produced in this case is shown in Fig. 6 which is a radiograph of the feet in Figs. 4 and 5.

Occasionally, much greater proportions and a more destructive character were noted; an example of this kind is given in Figs. 7 and 8. As in the preceding case, the lesion arose from the head of the fifth metatarsal but extended to the adjacent portions of the fourth metatarsal (Fig. 8); there was practically complete necrosis of the head of the fifth metatarsal followed by the formation of an irregular bony mass which is well shown in the radiograph (Fig. 8).<sup>1</sup>

A second group of lesions occurred along the shaft of the bone either singly or as multiple nodules. The majority of these were periosteal affections; they were usually located on the lateral or dorsal surface but were occasionally found on the plantar surface or completely encircled the bone. The usual form was that of a fusiform swelling such as is shown in Fig. 9. A more pronounced case of a similar character is seen in Fig. 10. In this instance, there were two distinct lesions present, one of which appeared as a spherical mass embracing the head of the metatarsal and the other as a fusiform enlargement extending the full length of the shaft. The condition was bilateral and symmetrical.

<sup>1</sup> Fig. 16 is taken from the same animal.

None of the periosteal lesions of the shaft thus far examined have resulted in a gross alteration which amounted to more than a slight erosion of the bone or slight roughening and thickening which persisted for some time after the healing of the lesion (see Fig. 21). Microscopic changes, however, were more marked.

In a few instances, affections of the bone or medullary cavity were discovered by x-ray examinations and verified by autopsy, but very little is known of their clinical history. At times, they produced conditions analogous to those of periostitis or resulted in necrosis and pathological fracture.

A third group of lesions arose from the distal end of the metatarsal, and while many of these were simple periosteal processes analogous to those of the shaft and the proximal end of the bone, they were of peculiar interest on account of the frequent occurrence of pathological fractures or epiphyseal separations. At present, we cannot attempt to differentiate between the various types of pathological processes which were concerned in the production of these conditions. Some were obviously the result of periosteal infections, while others appeared to be due to infection within the bone itself or to a combination of the two processes. An example of each of these may be given.

The lesion which is illustrated in Figs. 11 to 13 began as a simple periosteal process, and the first radiograph showed little if any alteration in the bone. Growth was unusually active, however, and within a week, slight crepitus could be detected and the radiograph showed the presence of a fracture of the neck just proximal to the epiphyseal line (Fig. 13). It could not be determined whether this was due entirely to the lesion of the periosteum or to the presence of a second focus of infection within the bone which was obscured by the periosteal lesion. When the animal was killed 11 days later, the bone was found to be irregularly and markedly thickened in its outer third.

The second case, illustrated in Figs. 14 and 15, is what appears to be a clear instance of an epiphyseal infection, or an osteochondritis, and is of especial interest on account of its occurrence in an adult animal. When first noted, there was a firm narrow ridge at the epiphyseal line which was barely palpable and could not be diagnosed with certainty as a syphilitic lesion. This ridge increased slightly, and within a few days, an edematous swelling appeared, the extent of which is indicated by the shadows in the radiograph taken at this time (Fig. 14). There was a distinct crepitus, and the radiograph showed an obliteration of the epiphyseal line with a clean-cut separation of the epiphysis (Fig. 14). The lesions healed with a slight bony thickening, and it will be noted that the epiphyseal line was not reestablished (Fig. 15).



From the description of metatarsal lesions which has been given, it will be seen that the essential differences between affections of these bones and those of the tibiofibular or ulna and radius were the extent of the bone involvement and the occurrence of focal infections within the bone and especially at lines of epiphyseal union.

*Tarsus.*—The status of syphilitic affections involving the tarsus is still somewhat obscure. As was pointed out in Part 1 of this paper (1), there is a variety of conditions affecting this part of the body which present much the same clinical picture. Those observed were chiefly affections of the calcaneus and included a few cases of syphilitic periostitis with numerous instances of necrosis of the calcaneus, pathological fracture, and epiphyseal separation together with some instances of extension of the infection to other bones of the tarsus or to ligaments, tendons, and synovial membranes.

All the periosteal lesions of the calcaneus were situated on the dorsal or superior surface of the bone near the anterior end. Normally, there is a well marked depression at this point which, in cases of periostitis, was obliterated or filled by a firm elastic mass. The resulting alteration in appearance is shown in Figs. 11 and 12, which may be compared with other illustrations.

So far as we are aware, in no instance was any marked necrosis of the underlying bone produced, but it is important to note that the majority of the cases of necrosis of the calcaneus which occurred in the absence of any known lesion of the periosteum occupied exactly the same position. This is clearly illustrated in Fig. 16.

The conditions designated as necrosis of the calcaneus, pathological fracture, and separation of the epiphysis have been considered in detail elsewhere (1). It seems necessary only to recall that all presented a common symptom-complex, the outstanding features of which were the sudden development of lameness, an edematous swelling of the tarsus, tenderness, and crepitus, the cause for which could be determined with accuracy only by radiograph or by careful dissection. The swelling and edema are well shown in Fig. 16.

In a few instances, other bones than the calcaneus have been found to be involved in affections of the type described and as a result of extension of lesions from such points as the head of the metatarsals (Fig. 16). However, they presented no distinctive clinical characteristics.

*Phalanges.*—As one passes from the larger and more accessible bony structures to those which are extremely small or difficult of examination, the question of the presence or absence of bone lesions and the nature of the injury become less certain. This applies to syphilitic affections of the phalanges and the bones of the carpus and tarsus as well. However, numerous instances of periostitis of the phalanges, or dactylitis, and of necrosis of the bones with no demonstrable lesion of the periosteum have been observed in the toes of the hind feet.

The occurrence of affections of the terminal phalanges which gave rise to abnormalities of the nails was referred to in connection with the cutaneous manifestations of syphilis (2, 3).

Lesions of this type are shown in Figs. 17 to 19 and also on the fifth toe in Fig. 8. There is a periostitis of the terminal phalanges of several of the toes in Figs. 17 and 18, and the loss of the nail can be traced in the fourth toe of the left foot. Fig. 19 shows a more extreme condition, which was permanent. Enlargement and induration of the terminal phalanx, as in these cases, rarely occurred without the production of an easily recognizable paronychia.

Recorded instances of a simple periostitis of the phalanges which did not involve the skin or its appendages were comparatively few. However, a large proportion of these cases occurred on the fifth or lateral toe as in Figs. 20 and 21, thus conforming to the order of distribution recorded for other lesions of the hind feet. In this connection, attention may be called to a striking tendency towards the occurrence of serial periosteal lesions along the outer sides of the feet, producing a beaded effect which is well shown in Fig. 21.

Lesions were observed on the first, second, and third phalanx; some produced no appreciable alteration in the bone, while others resulted in necrosis which was occasionally followed by fracture or disintegration.

Infections arising within the bone were discovered in a few animals, and their development was followed by serial radiographs. There were no appreciable external evidences of bone involvement in these cases until necrosis and disintegration or fracture of the bone occurred. The parts then became more or less swollen and tender, and in some instances a crepitus could be detected.

Several examples of affections of this kind may be seen in Figs. 16 to 18. The lesion in the first phalanx of the outer toe of the right foot in Figs. 17 and 18 is brought out especially well. By careful inspection (use reading glass), it will be seen that there is an enlargement of the distal end of this phalanx (Fig. 17) with rarefaction and loss of architecture, but that there is no shadow to indicate the presence of a periosteal lesion. 13 days later (Fig. 18), a fracture occurred at this point accompanied by the usual reaction in the surrounding tissues.

There were a number of definite bone lesions present in the feet of this animal, none of which could have been clearly recognized except by radiographic examination; there were periosteal lesions of several toes (outwardly paronychias), and osteitis in the phalanx mentioned and at both extremities of the fifth metatarsal of the same foot. In Fig. 16, a number of the phalanges are also seen to be involved. This will serve to indicate the difficulty in the recognition of lesions of this type, our knowledge of which is at present rather limited.

### *Obscure Bone Affections.*

The conditions which have been described in the preceding pages may with reason be regarded as the obvious bone affections in contradistinction to infections which are accompanied by no signs which are sufficiently distinctive to enable one to recognize them clinically or even to suggest the possibility of their existence. It is quite certain that such conditions do occur as indicated by the frequency with which affections of the interior of the nares and of the small bones of the feet have been discovered by radiographic or pathological examination.

Other positions in which we have reason to believe that obscure affections may exist are the vertebral column, the cranial bones, the sternum, the ribs, and the mandibles. In each of these sites, lesions have been found whose pathology was sufficient to identify them as syphilitic. Infection of the caudal vertebræ has been recognized clinically in several animals, the condition being manifested as fusiform swellings. In other instances where no injury was suspected, necrosis of the bones was demonstrated by radiograph.

Our attention was attracted to two cases of infection of the cervical vertebræ by the development of symptoms of meningitis. In one of these animals, postmortem examination revealed the presence of necrosis of the cervical vertebræ, and spirochetes were demonstrated in the cerebrospinal fluid. In the other, the upper cervical vertebræ

and one of the occipital condyles were involved. Histologically, these bones showed a typical syphilitic reaction in the Haversian canals and marrow spaces, with absorption and necrosis of bone.

In two instances, lesions were discovered at autopsy at the costochondral junction of one of the ribs which corresponded grossly and histologically with other bone lesions in the animals. In another animal, a healed lesion was found in the xiphoid and the adjacent part of the body of the sternum which appeared to be of syphilitic origin, and a similar condition was observed in the mandible of another animal.

The occurrence of obscure bone affections and infections associated with very slight local reaction is of considerable importance and lends support to the belief that many cases of latent or obscure human infection may find their explanation in the occurrence of an analogous group of conditions.

#### *Tendons and Synovial Membranes.*

In Part 1 of this paper (1), mention was made of the occurrence of affections of tendons, joints, and synovial membranes. With the exception of the tendons, these conditions were not accompanied by signs which were sufficiently distinctive to permit their clinical recognition, and nothing can be said of their clinical history.

Only a few cases of primary tendon involvement were recognized clinically, all of which occurred in the tendo achillis. The lesions were usually small nodular, or fusiform masses which could be detected only by palpation. In one instance, however, the lesion developed on the outer side of the tendon and produced a visible enlargement over which the skin was freely movable.

#### *Clinical History of Bone Affections.*

The clinical history of bone affections cannot be given in so complete form as one would wish, since only a few animals of this group were held for any considerable period of time; our knowledge is, therefore, chiefly of the early stages of the infection.

From available data, it would appear that syphilis of the bones occupies a position with relation to the infection as a whole which is closely analogous to that of the cutaneous manifestations.

Among conditions which seemed to exercise a predisposing influence upon the occurrence of bone infection, mention may be made of the reduction or early suppression of primary lesions by unilateral instead of bilateral inoculation, by castration or excision of lesions, or by the use of therapeutic agents. When procedures of this kind were employed, the relative incidence as well as the extent of the bone involvement was definitely increased. The peculiarities of distribution and the marked predilection for unprotected bony prominences would indicate that the factor of trauma also plays a part both in the occurrence and in the localization of this class of lesions.

The important point to be noted, however, is that lesions of periosteum and bone appear to occupy a definite position in the scheme of defensive reactions in the experimental animal. Initially, this reaction is bound up with the development of the primary lesions, but if the latter are suppressed at an early stage of the infection, it is almost invariably taken up by the periosteum and bone. Conversely, if the primary lesions are allowed to progress uninterruptedly, or if they are suppressed late in the course of the infection, protection is apt to be conferred upon this group of tissues, and the burden of reaction is assumed by some other set of tissues such as the skin or the mucous membranes. The detailed consideration of the facts upon which this statement is based will be taken up elsewhere (4).

*Time of Occurrence.*—From what has been said, it may be surmised that the time of appearance of bone lesions varied greatly. Under conditions which favored their development, they were among the earliest manifestations of a generalized disease. While the extreme limits of variation in our series of animals were from a minimum of 38 days to 15½ months after inoculation, most often the lesions appeared within 2 to 3 months. The reactive period of bone infection, if we may term it such, was shorter and more sharply demarcated than that of other lesions. This was especially true in animals whose primary lesions had been suppressed. In these, the lesions appeared with great regularity at or near the expiration of the 60 day period following inoculation, those of the nasal region as a rule being recognized slightly earlier than those of other bones.

*Course and Duration.*—The course of the bone lesions was one of comparatively rapid development and rapid decline, in which there

was less of the cyclic variation to be seen than in almost any other class of syphilitic affections. Clearly defined periods of growth and regression were observed in some animals, but it is worthy of note that no instance of true relapse or recurrence of a healed lesion was recorded. In a single instance, in which a periosteal lesion on the nose of an animal persisted for more than a year, there were several periods of marked regression followed by renewed activity; this was the nearest approach to true relapse which was observed. Whether these peculiarities of bone lesions may be attributed to difficulties of observation on the one hand and, in our series, to the short period of observation on the other, or are characteristic of this group of affections cannot be said.

As a rule, the period of active infection was comparatively short. Some lesions disappeared within a few weeks, and few lasted for more than 1 to 2 months. The process of repair in cases of marked bone destruction was obviously more prolonged. In exceptional instances, active lesions persisted for several months; the longest recorded period in our series was 20 months, and this lesion was still active when the animal was killed.

Gross evidences of bone injury were, as a rule, comparatively slight and tended to disappear; in the case of the more destructive lesions, however, repair was less perfect and more or less permanent alterations were produced which were akin to those seen in syphilitic infections of man.

#### CONCLUSIONS.

The facts presented above indicate clearly that localized infections of periosteum, bone, cartilage, tendons, and synovial membranes form an important group of affections in rabbits inoculated with suitable strains of *Treponema pallidum*.

Among the more important features of this group, mention may be made of the early occurrence of the lesions and their relation to other manifestations of disease, the involvement of bones in exposed locations or of bones which are subject to pressure or trauma, the high incidence and destructiveness of affections of the nasal region, the tendency to localization at points of bony union or at epiphyseal lines, and the frequency of obscure focal lesions.

From a general standpoint, it will be seen that syphilis of the skeletal system of the rabbit is a very characteristic condition and that it combines features of both the acquired and the inherited form of the disease in man. It cannot be said that the two conditions are identical, yet they possess many fundamental characteristics in common, and it is believed that this form of experimental syphilis is of more than usual importance on account of the opportunity which it affords for the study of lesions of the skeletal system.

#### SUMMARY.

A systematic study was made of the affections of bone, cartilage, tendons, and synovial membranes which occurred in a series of rabbits with generalized syphilis. Localized infection of this group of structures was found to be of very frequent occurrence. The parts involved were, in the order of their frequency, the facial and cranial bones and cartilages, the bones, tendons, and joints of the feet and legs, the cervical and caudal vertebræ, the ribs, and the sternum. These infections often gave rise to characteristic manifestations of disease which could be detected without difficulty by inspection or palpation of the part. In many instances, however, clinical manifestations were so slight that the presence of lesions could be detected only by radiographic or pathological examination.

Detailed descriptions of various clinical types of disease were given and the clinical manifestations correlated with the pathological process. It was pointed out that bone lesions exhibited a decided predilection for certain exposed bony prominences, for lines of bony union, and for epiphyseal lines in particular.

A study of the clinical history of bone lesions brought out the fact that they were among the earliest of the generalized forms of disease; they tended to pursue a comparatively rapid course, and relapse was never observed.

Especial emphasis was laid upon three aspects of the experimental infection: the analogy existing between certain forms of the animal and human affections, the relation of syphilis of the osseous system to other evidences of disease, and the occurrence of obscure bone lesions.

In this connection, it was pointed out that the nasal and epiphyseal lesions of the rabbit presented a striking analogy to those of congenital syphilis in man.

It was also pointed out that syphilis of the osseous system occupied a definite position in the scheme of defensive reactions such that lesions of these tissues might be favored or inhibited according to the experimental conditions employed.

Finally, the frequency with which infections occurred which were not accompanied by sufficiently distinctive signs even to suggest the possibility of their existence was interpreted as evidence that some cases of latent or obscure infection in man might find their explanation in the presence of a similar group of affections.

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#### EXPLANATION OF PLATES.

All illustrations are reproductions of photographs or radiographs which have not been retouched and represent objects at their natural size. Statements of time are estimated from the date of inoculation unless otherwise indicated.

#### PLATE 67.

FIGS. 1 to 3. Syphilitic affections of the tibiofibular, or external malleolus.

FIG. 1. 84 days. A typical case of bilateral, nodular periostitis of the external malleolus.

FIG. 2. Autopsy specimen of the left leg showing the almost spherical granulomatous mass with some extension to the surrounding tissues.

FIG. 3. Radiograph showing location of the lesions in Fig. 1 with reference to bony parts and the apparent effect upon the underlying bone. Note the slight alteration in the margin of the shaft of the left tibia as compared with the right. The most marked changes are in the head of the bones (indicated by arrows). The lighter shadows in the left tibia are possibly due to lesions within the bone.

FIG. 4. Appearance of normal metatarsal.

FIGS. 5 to 15. Syphilitic affections of the metatarsals.



## PLATE 68.

FIGS. 5 to 8. Lesions of the proximal end of the metatarsals.

FIG. 4. Lateral view of right hind foot showing normal topography of the metatarsal area.

FIG. 5. 135 days. Left foot of same animal showing a typical enlargement of the head and adjacent portions of the shaft of the fifth metatarsal due to syphilitic involvement of this portion of the bone.

FIG. 6. Radiographs of the feet shown in Figs. 4 and 5. Note the comparative condition of the proximal portions of the lateral metatarsals.

FIG. 7. 83 days. A marked syphilitic affection of the proximal ends of the fourth and fifth metatarsals.

FIG. 8. Radiograph showing the extent of the bone involvement.

## PLATE 69.

FIGS. 9 to 13. Affections of the shaft and distal ends of the metatarsals.

FIG. 9. 117 days. A fusiform lesion of the shaft of the lateral metatarsal. Periostitis.

FIG. 10. 93 days. A marked periosteal affection of the entire fifth metatarsal. There is a nodular granulomatous lesion at the proximal end of the bone and a fusiform lesion extending its full length; this lesion completely surrounded the bone. There is also a skin lesion over the tarsus and just above this, a periosteal lesion of the tibiofibular.

FIGS. 11 and 12. 92 days. Dorsal and lateral views of a marked affection of the distal end of the fifth metatarsal. These photographs also show a prominent periosteal granuloma on the dorsal surface of the anterior end of the calcaneus (marked by arrow).

FIG. 13. Radiograph showing pathological fracture of the shaft of the metatarsal resulting from the lesion shown in Figs. 11 and 12.

## PLATE 70.

FIGS. 14 and 15. Affections of the distal ends of the metatarsals.

FIG. 16. Affection of the tarsus.

FIG. 14. 58 days. An epiphyseal separation of the distal end of the fifth metatarsals. A displacement of the shafts and epiphyses is seen; a light line or band is seen crossing the bone in the position of the epiphyseal line, and a small spur of bone projects from the outside of both metatarsals. Lesions marked by arrows.

FIG. 15. 6 weeks later. Repair of the epiphyseal lesions. Note the general enlargement of the distal ends of the bones (lateral metatarsals), the loss of epiphyseal lines, and the thickening of the bones at the site of the lesions. Adjacent areas of the fourth metatarsals also appear to be slightly altered.

FIG. 16. 84 days. An unusually pronounced case of bone syphilis. There is necrosis of the right calcaneus associated with effusion into the surrounding tissues and swelling of the tarsus, foot, and leg. Necrosis of the left lateral metatarsal is also present, with effusion and swelling of the foot and tarsus, and a number of lesions may be seen in the phalanges of both feet (marked by arrows). Pathological fracture of several of these bones occurred a few days later.

FIGS. 17 to 21. Affections of the metatarsals and phalanges.

#### PLATE 71.

FIG. 17. 92 days. Periostitis and osteitis. Left foot: periostitis of all three phalanges of the fifth toe and of the second and third phalanges of the fourth toe. Right foot: periostitis of the terminal phalanges of the third toe and of the shaft of the lateral metatarsal; osteitis of the proximal and distal ends of the metatarsal (note absence of epiphyscal line) and of the distal end of the first phalanx of the fifth toe. Other shadows are from cutaneous lesions.

FIG. 18. 2 weeks later. Left foot: necrosis and buckling of the second phalanx of the fifth toe and loss of nail of the fourth toe. Right foot: necrosis and fracture of first phalanx of fifth toe.

FIG. 19. 1 year and 9 months. Marked permanent lesions of the phalanges.

#### PLATE 72.

FIGS. 20 and 21. Symmetrical affections of the lateral metatarsals and phalanges showing distributional tendencies.

FIG. 20. 85 days. Periosteal lesions on the lateral surface of the feet extending from the base of the metatarsals to the terminal phalanges. Other toes are slightly affected, notably the fourth toe of the left foot.

FIG. 21. 85 days. Serial lesions of the metatarsals and phalanges illustrating the bead-like effect produced in the rabbit's foot and various degrees of bone involvement associated with them.<sup>2</sup>

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<sup>2</sup> See also Brown, Pearce, and Witherbee (1), Fig. 3.

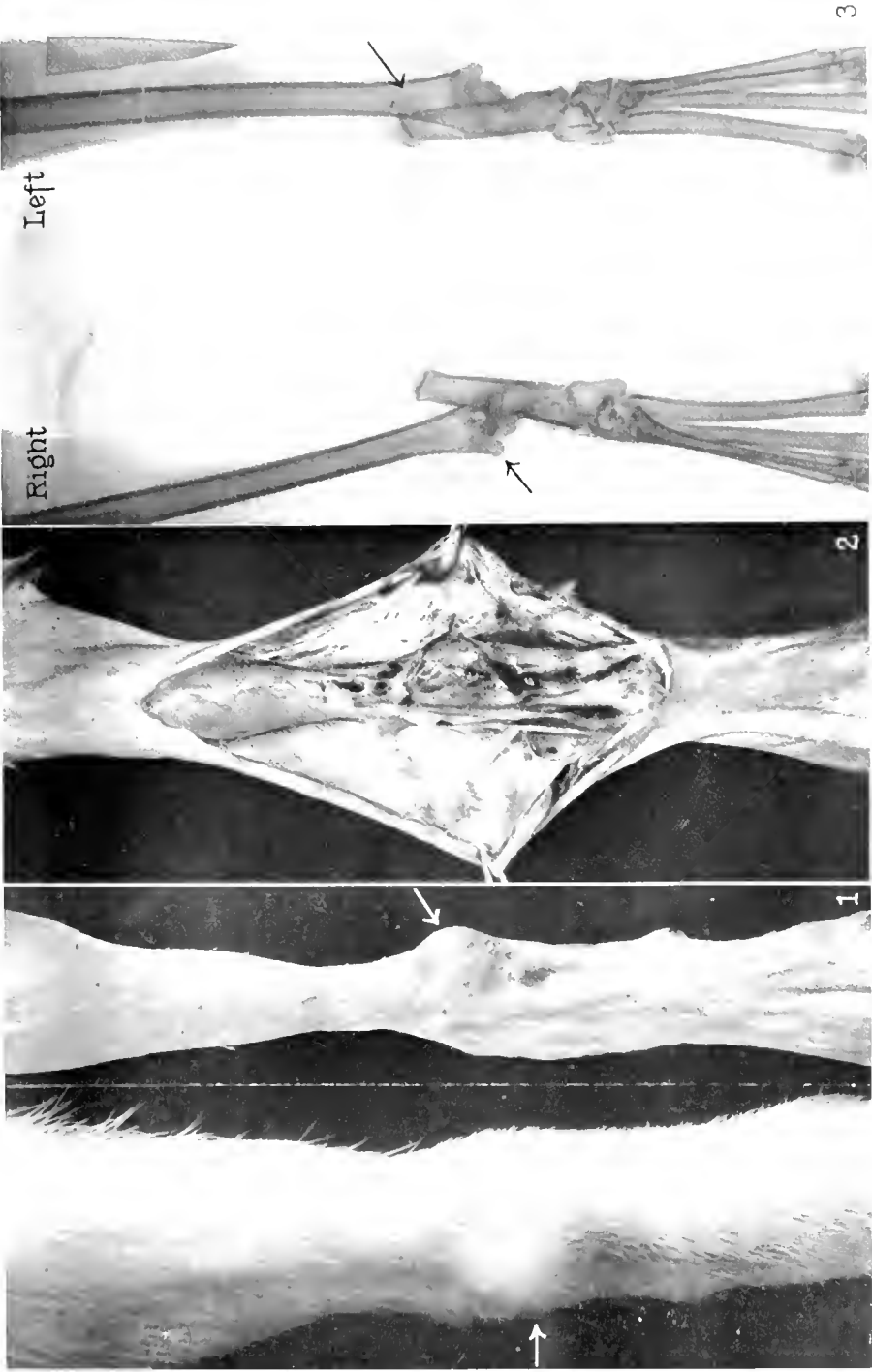
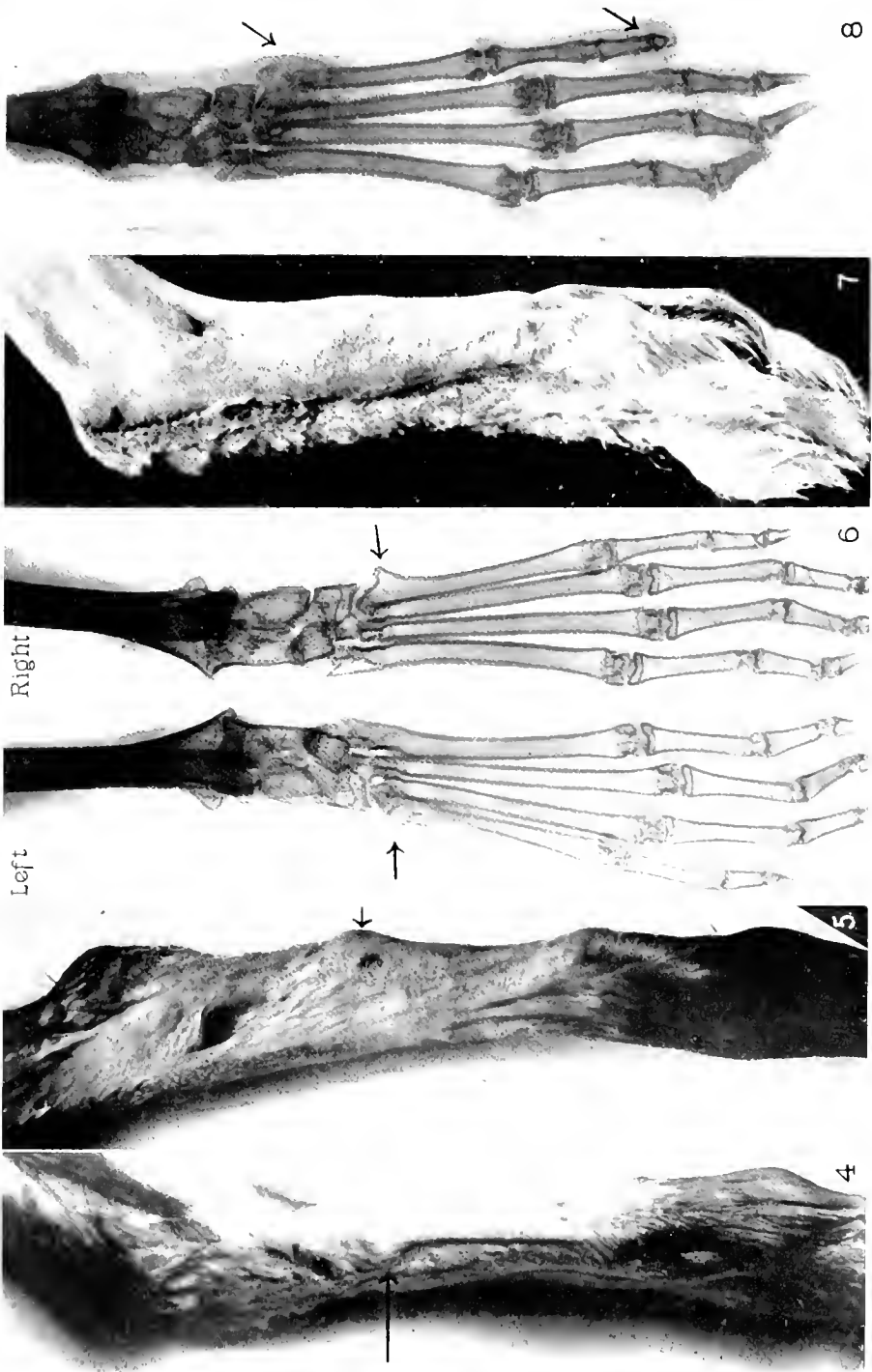


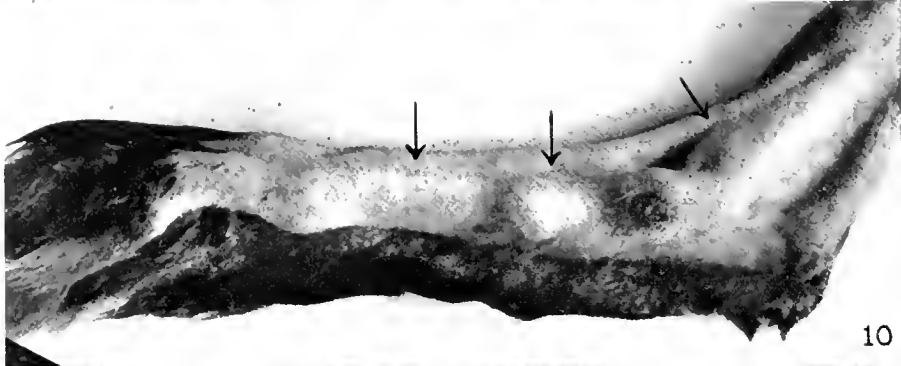
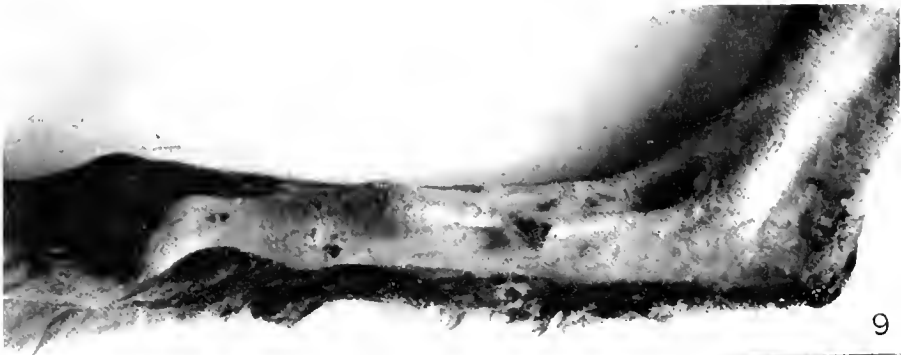
FIGURE 1. Pearce and Witherby. Experimental cyphosis in the thoracic spine.





(Brown, Pearce, and Witherbee. Experimental syphilis in the rabbit. VI)

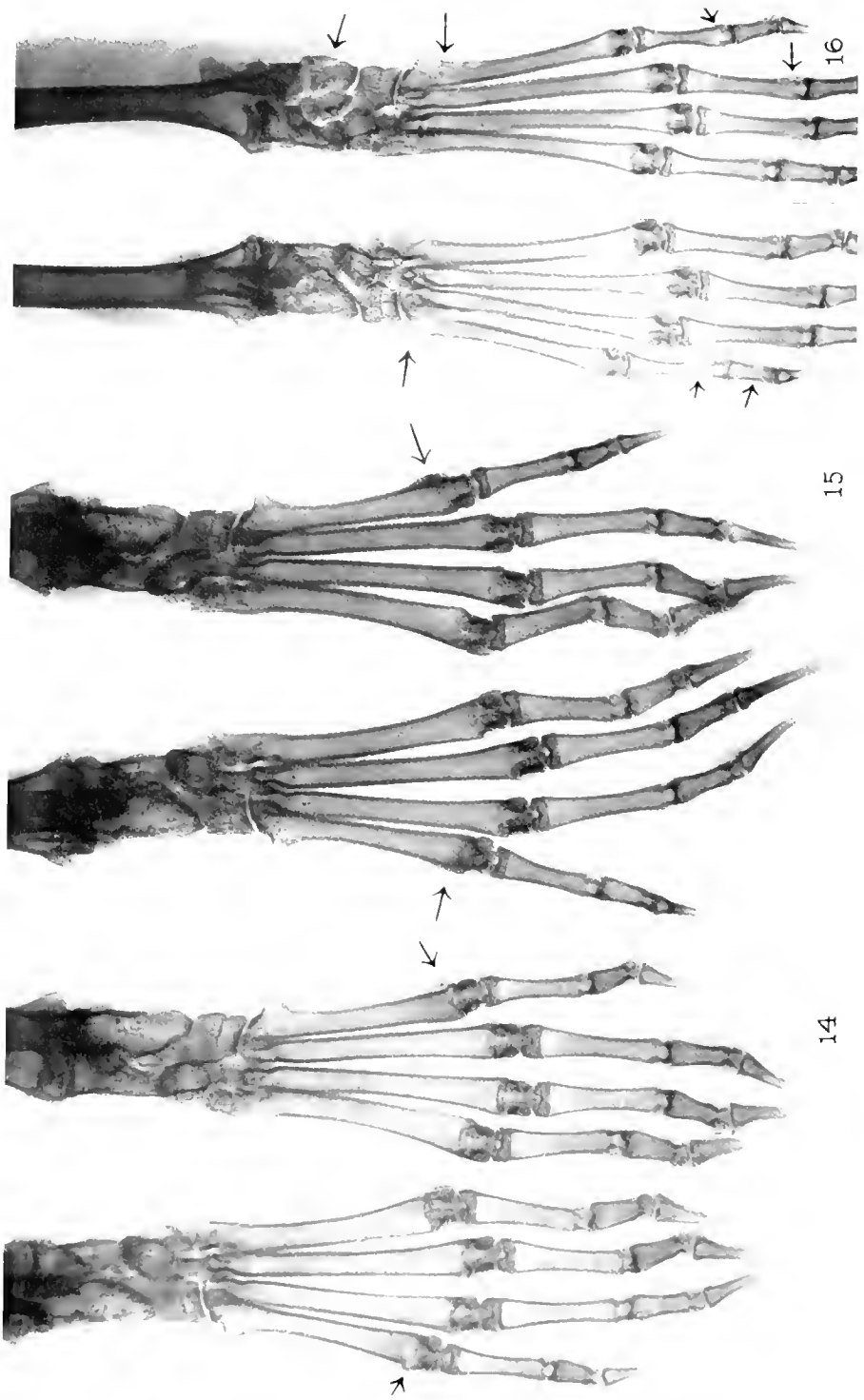




(Brown, Pearce, and Witherby: Experimental syphilis in the rabbit. *AMJ*)



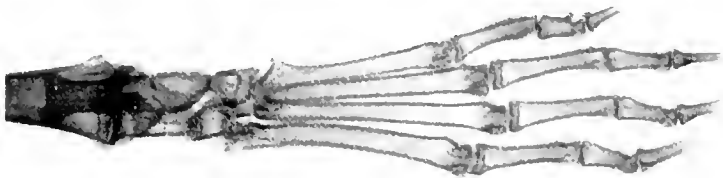




(Brown, Pearce, and Witherbee. Experimental syphilis in the rabbit. VI.)



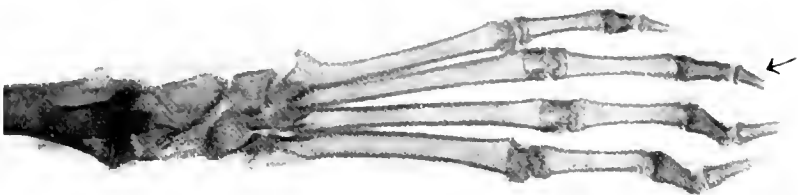
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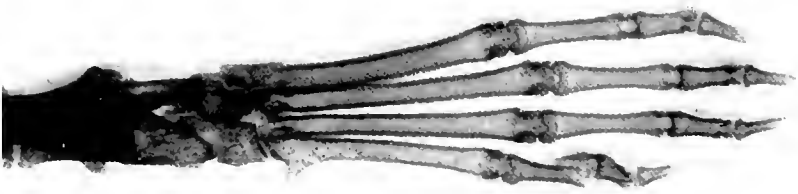
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Left



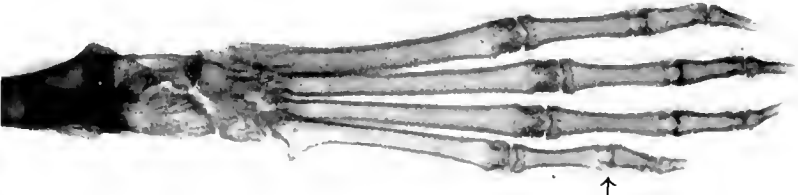
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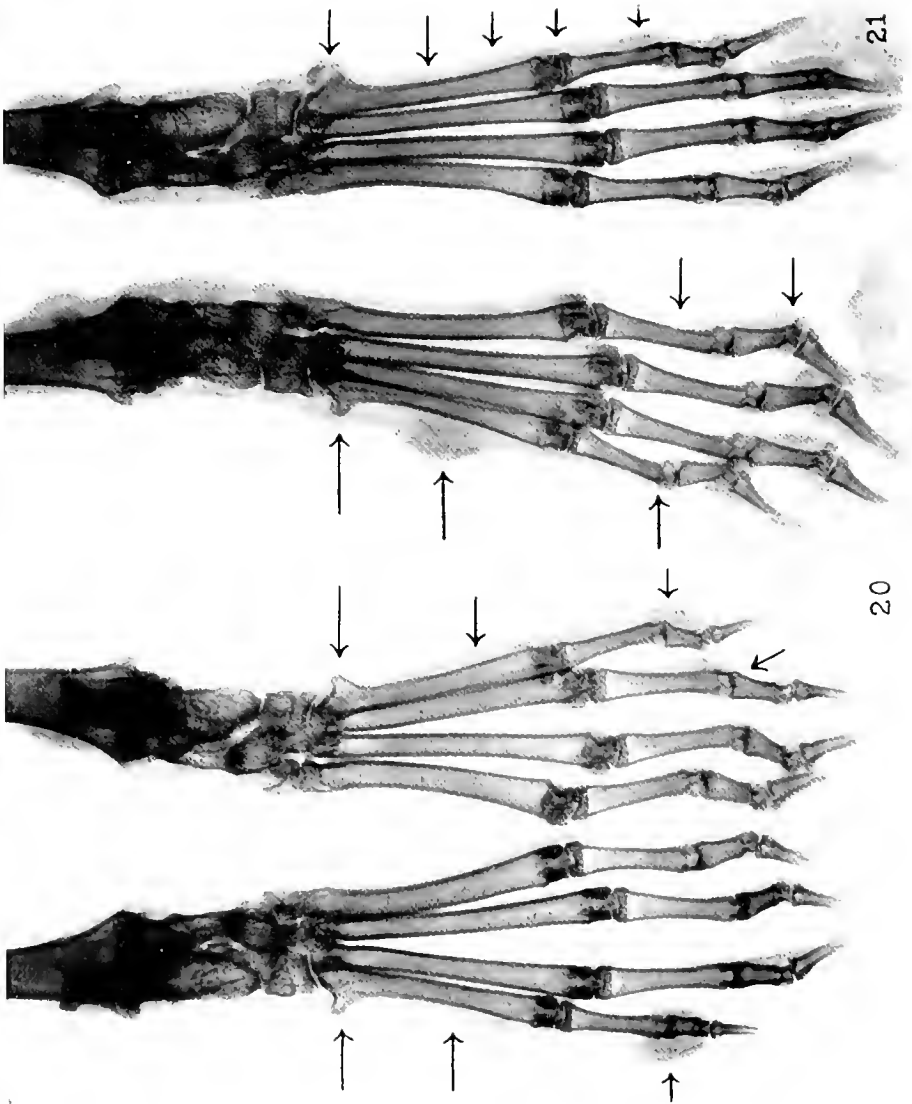
Left



Right

(Brown, Pearce, and Witherby: Experimental syphilis in the rabbit. A1.)





(Brown, Pearce, and Witherell. Experimental syphilis in the rabbit. VI.)



## A NEW STRAIN OF TRANSMISSIBLE LEUCEMIA IN FOWLS (STRAIN H).

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(Received for publication, November 11, 1919.)

In previous reports (1915, 1918) the writer has described three types of leucemia in the fowl, the lymphatic, the myeloid, and the intravascular lymphoid<sup>1</sup> types. Reference has also been made to purely anemic forms, which appear to be closely related to the intravascular lymphoid type. Within one type the clinical picture of the disease presents many variations, which recall analogous instances in man. The association with neoplastic growths—lymphomata and myeloid tumors—is noteworthy. In previous experiments the author has demonstrated that the various types of leucemic disease may be produced by the same experimental strain. Hence the conclusion seems justified that all are produced by the same virus.

The study here reported, with a new strain, was undertaken in order to verify previous findings and to elucidate the histogenetic processes. It deals with general considerations relating to the strain, particularly the problem of virulence. The strain employed was derived from a hen with leucemia received on February 22, 1917, from Professor Carl Hansen.

*Protocol 1.*—Hen H. February 22, 1917. Color of comb pale. Hemoglobin 20 per cent (Sahli). Blood films show well pronounced leucemia. Every microscopic field reveals myelocytes, but lymphoid cells predominate. These cells have dark, round nuclei and a scanty border of protoplasm. There is

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<sup>1</sup> The term intravascular lymphoid leucemia is merely tentative. In this type, which is closely related to the pure anemic form, microscopic examination shows only intravascular deposits of lymphoid cells, which are probably erythroblasts in early stages. The type of disease must be regarded as a severe anemia with an excessive pathological regeneration. An article on this subject will appear shortly in *Folia haematologica*.

often marked basophilia. Mitotic divisions are numerous. There are few typical, small lymphocytes, polynuclear cells, and large mononuclear cells to be seen. Erythroblasts are found frequently. The oxidase reaction reveals blue granules in some myelocytes, but the granules in most of these cells are unstained. Erythrocytes 1,080,000; leucocytes 720,000. The differential count of leucocytes is given in Table I.

February 24, 1917. Animal killed.

TABLE I.  
*Differential Leucocyte Count of Hen II.*

Cells.	Per cent.	No.
Polynuclears.....	2	14,400
Myelocytes.....	15	108,000
Large mononuclears.....	4	28,800
Lymphocytes.....	4	28,800
Lymphoid cells.....	75	540,000

*Autopsy.—Liver.*—Greatly enlarged; weight 156 gm. Color brownish, with numerous fine white spots on surface. These can barely be seen on the cut surface. *Spleen.*—Enormously enlarged; weight 40 gm. Bluish red in color. Cut surface quite uniform. *Kidneys.*—Brown with very fine white spots. Weight of right kidney 8 gm. *Heart.*—On anterior side shows a large white spot the size of a hemp-seed. *Lungs, stomach, intestines, thymus, bones, and skin.*—Show no pathological changes.

*Microscopic Examination.—Liver.*—Shows a number of medium sized periportal cell infiltrations, containing typical myelocytes. Non-granular cells are absent. The capillaries are markedly dilated and filled with large, round lymphoid cells and scanty myelocytes and erythrocytes. The cell masses produce complete injection of the capillary network. *Spleen.*—Only mere traces of follicles are seen. Pulp well filled with large lymphoid cells, which often show mitotic division. Perivascular layers of myelocytes are rarely encountered. *Bone marrow.*—The trabeculae are partly atrophic and contain small dark cells. Some are of normal size and contain myelocytes. Fat cells are reduced in number. *Kidney.*—Interstitial infiltration with myelocytes in the superficial cortical layer. Capillaries often full of large lymphoid cells. *Heart.*—The white nodule consists of large cells, with oval nuclei and large masses of protoplasm without granules. Typical myelocytes are seen. *Intestine.*—In the deeper portions of the mucous membrane of the colon are found myelocytes in thin layers.

The case is one of typical leucosis with leucemia. The blood picture is characterized by the large number of leucocytes (myelocytes and



large lymphoid forms). The histological examination shows marked filling of the capillaries of various organs with leucocytes (leucostasis) and interstitial deposits of myelocytes in the liver, spleen, and kidneys. The case represents a mixture of the myeloid and the intravascular lymphoid types of disease.

Eight normal hens were inoculated into the wing vein with citrated blood from the original fowl. One of the inoculated birds developed leucosis. The inoculations were then continued through twelve generations. In all, 122 birds were inoculated. In 34 instances (28 per cent) the inoculations gave positive results. For comparison it may be mentioned that Ellermann and Bang (1908, *a*, *b*, 1909) had 39 per cent positive results from Strain A; Hirschfeld and Jacoby (1910), 44 per cent; Ellermann (1918), 22 per cent from Strain E; and Schmeisser, 33 per cent. One constantly finds a certain percentage of birds immune. The resistance is not, however, constant, as by repeated inoculations it may be possible to transmit the disease to fowls originally immune. Both these facts were again confirmed by the experiments made with Strain H.

#### *Virulence of Inoculation Material.*

In a previous article (Ellermann and Bang, 1909) it was pointed out that the number of takes in a single experiment may vary to a large extent. Hence it is necessary to employ a considerable number of birds in order to obtain positive results. If, for example, only five fowls are used, it often happens (in about 25 per cent of the experiments) that the outcome is negative notwithstanding the high degree of virulence possessed by the inoculation material. By using a larger number of fowls, the chances of obtaining positive results are increased, and with as many as twelve, transmission of the disease is practically certain. For the sake of economy, the writer was limited to eight fowls for each transmission experiment, with, as a result, somewhat lessened chances of a successful outcome. None of the fowls may acquire the disease, or, on the other hand, all may become infected. In the latter instance, we should not be justified in drawing the conclusion that the virus had a high degree of virulence. This variability in infectivity is shown in Table II. It would be advantageous if

it were possible to increase the number of infected animals, either by lowering the natural resistance of from 60 to 70 per cent of the fowls or by augmenting the virulence of the inoculation material. Attempts to lower the resistance by bleeding or hunger, made in previous experiments, were unsuccessful. An experiment of this character was undertaken with the present strain. Before inoculation of the leucosis virus an injection of tuberculin was given in eight fowls. The disease developed rapidly, appearing 2 or 3 weeks after

TABLE II.  
*Number of Fowls Infected with Strain H.*

Generation.	No. of fowls inoculated.	No. of fowls infected.	Percentage infected.
1	8	1	13
2	20	7	35
3	8	5	63
4	16	2	13
5	16	3	19
6	8	1	13
7	8	3	38
8	6	1	17
9	8	4	50
10	8	4	50
11	8	2	25
12	8	1	13

inoculation, and the fowls died after a short interval—only 32 days. It has not been possible to repeat the experiment, but probably this result was accidental and can be explained in some other way.

A study of Table III, which gives the duration of illness in the successive generations of inoculated fowls, shows a progressive shortening of the period. At the beginning of the series the duration of the disease from the time of inoculation to death was 15 to 20 weeks, while at the end it was only 6 to 8 weeks. This shortening of the duration of the disease must be considered as attributable to increased virulence of the virus.<sup>2</sup>

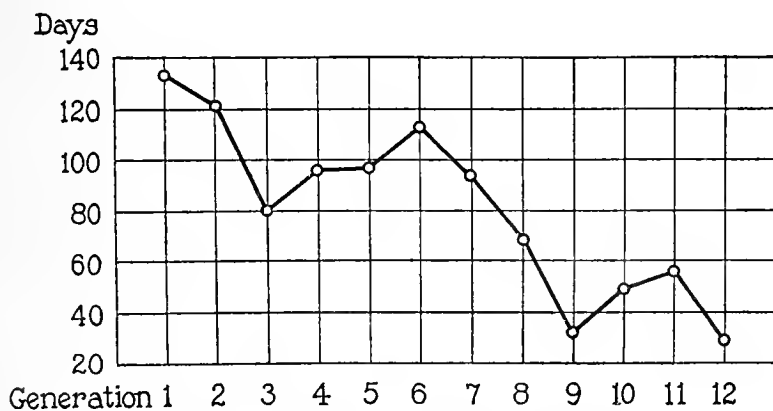
<sup>2</sup> Nothing can be stated concerning the true nature of the virulence, as different degrees of virulence may exist.

A graphic representation of the reduction in duration of the disease is given in Text-fig. 1. While the curve is not absolutely regular, the fall is distinct. The lack of regularity may be accounted for by

TABLE III.

*Duration of the Disease with Strain H from the Time of Inoculation to Death.*

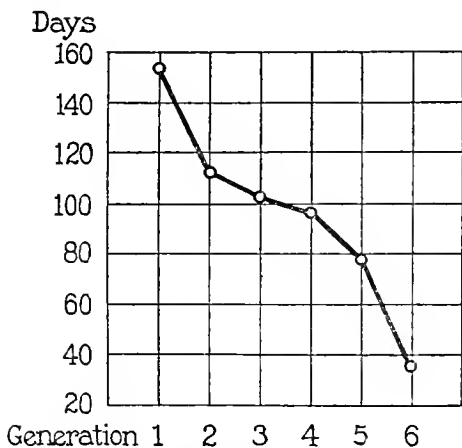
Generation.	Duration of disease.
	days
1	>133
2	121
3	80
4	96
5	97
6	113
7	94
8	69
9	32
10	49
11	56
12	29



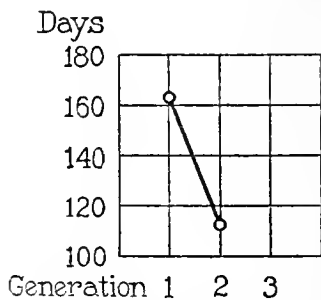
TEXT-FIG. 1. Duration of the disease with Strain H.

the irregularity in the sixth generation, caused, however, by only a single case. The fall in the ninth generation, in which the fowls had been treated with tuberculin previous to inoculation, is not very striking in comparison with the preceding part of the curve.

Text-figs. 2 and 3 are based on former experiments made with Strains E and D. It will be observed that these curves are similar to that of Text-fig. 1. The curve of Text-fig. 2 is very regular. By comparing the three curves it will be seen that the duration in the first generation is about the same for the three strains. It must be added that the quantity of material inoculated varied somewhat in the different experiments. As a rule, however, the amount consisted of a blood suspension corresponding to 2 drops of blood of the sick fowl. Sometimes 1 cc. of a centrifuged tissue emulsion was used. A study of this point shows that the variations in the curves



TEXT-FIG. 2. Duration of the disease with Strain E.



TEXT-FIG. 3. Duration of the disease with Strain D.

cannot be explained on the basis of the quantity inoculated. Hence it is possible to increase the virulence of the virus of leucosis by passage. This finding suggests similar results obtained with another filterable virus; namely, that of hydrophobia. By inoculating the street virus of a spontaneous case the incubation period decreases with each generation, until it reaches a fixed point. The increased virulence of the virus of leucosis used in the fowl is shown by a shortening in the duration of the illness. It is noteworthy, however, that there is no coincident rise in the number of successful infections. It appears doubtful whether any increase in the number of infections can be obtained through increased virulence of the virus.

The shortening of the duration of the disease with continued passage was partly overlooked in former experiments, and partly explained in another way. It was believed that the duration was determined by the type of the disease; that is, that the intravascular lymphoid cases had a shorter duration than the lymphatic cases. This viewpoint cannot, however, now be maintained. The main factor, apparently, is the position of the individual case in the series of generations. Hirschfeld and Jacoby (1910) were correct in their assertion that the length of the incubation period is an indicator of the degree of virulence. With Strain A of Ellermann and Bang, which had required an incubation period of about 8 weeks in the first generation, they observed an incubation period of 6 weeks at the beginning of their series, which later decreased to 4 weeks. The conclusions of Hirschfeld and Jacoby seem, however, to be based on a very small series of observations, and the use of the incubation period as an indicator of virulence has disadvantages. For instance, the time of onset of the disease cannot be established accurately if the onset is gradual or the course intermittent. In the lymphatic cases we cannot use the term incubation period, inasmuch as anemia and blood changes are usually absent.

*Types of Disease.*—It was observed with Strains D and E that the type of the resulting disease appeared to vary. Strain D, which originated from an intravascular lymphoid case, produced in the first generation a myeloid, and in the second generation a lymphatic case. From Strain E, originating from a lymphatic case, a series of lymphatic and intravascular cases, and also a few myeloid cases, resulted.

Table IV shows the results of experiments with Strain H, which was obtained from a case of mixed myeloid-intravascular character. In the first eleven generations we find myeloid, intravascular lymphoid, and anemic cases, while in the twelfth generation a lymphatic case suddenly appears. Table V gives the number of cases of each type.

The observations made on Strain H confirm the finding of a variation in the type of disease. The clinical pictures are varied, and intermediary forms difficult to classify are also present. A change from the myeloid to the lymphatic type, or the reverse, is rare. Caution

is necessary in diagnosing cases, and the possibility of occasional spontaneous cases must be kept in mind. Each strain seems to have a tendency to produce a certain type of disease. Strain E, for instance,

TABLE IV.  
*Types of Disease Obtained with Strain H.*

Generation.	Types of cases.
1	Intravascular (No. 1).
2	Anemic (No. 2); intravascular (Nos. 3, 4, 5, and 7); myeloid (No. 6); myeloid and intravascular (No. 8).
3	Intravascular (Nos. 9 and 14); myeloid (Nos. 10 and 11); anemic (Nos. 12 and 13).
4	Anemic (Nos. 15 and 19); myeloid (Nos. 16 and 17); intravascular (No. 18).
5	Intravascular (Nos. 20, 21, and 22).
6	" (No. 23).
7	" (Nos. 24 and 25); myeloid (No. 26).
8	" ( " 27 " 28).
9	" ( " 29, 30, and 32); myeloid (No. 31).
10	" ( " 33 and 35); myeloid (Nos. 34 and 36).
11	" (No. 37); myeloid (No. 38).
12	Lymphatic (No. 39).

TABLE V.  
*Number of Cases of Different Types Produced by Strain H.*

Type of case.	No.
Intravascular and anemic.....	27
Myeloid (leucemic).....	9
" (aleucemic).....	2
Lymphatic.....	1
Total.....	39

produces mainly intravascular and lymphatic, and Strain H chiefly intravascular and myeloid cases. It appears, then, that the same virus at times produces a myelotropic and at other times a lymphotropic effect. Further investigation is needed to elucidate this point.

*Serological Properties of Different Types.*

An attempt to find isohemolysins or isoagglutinins gave negative results. On the other hand, it was found that the hemolytic power of the serum against rabbit erythrocytes was diminished in a fowl showing anemia. While normal fowl serum dissolved rabbit erythrocytes in about 6 minutes, it required a considerably longer period to produce hemolysis with serum from the anemic fowl. The conditions of the experiment were identical in both cases.

TABLE VI.  
*Hemolysis in Cases of Intravascular Lymphoid Leucosis.*

Fowl No.	Date.	Hemoglobin (corrected).	Length of time required for hemolysis.	Length of time required for hemolysis in normal serum control.
	1917	per cent	min.	min.
5	Sept. 25	30	15	5
	Oct. 7	42	14	8
7	" 1	75	16	8
	" 17	58	12	5
3	" 3	33	20	8
	" 17	25	10	5
13	" 27	25	10	6

TABLE VII.  
*Hemolysis in Cases of Myeloid Leucosis.*

Fowl No.	Date.	Hemoglobin (corrected).	Length of time required for hemolysis.	Length of time required for hemolysis in normal serum control.
	1917	per cent	min.	min.
8	Oct. 4	42	10	8
6	Nov. 1	33	6	6
17	" 3	33	5	7

The technique employed was that described below. Rabbit blood was mixed with one-tenth of its volume of a 10 per cent solution of sodium citrate. After centrifugation, the blood corpuscles were used directly, without washing, for making a 3 per cent suspension in 0.9 per cent solution of sodium chloride. The fowl serum was prepared by keeping the blood in a cool room for 24 hours. 0.1 cc. of blood suspension was

added to 0.2 cc. of the active serum by means of a capillary pipette in such a manner as to produce an instantaneous mixture.<sup>3</sup> Tables VI and VII give the results of these experiments. The former comprises four cases of intravascular lymphoid leucosis. It will be seen that in comparison with normal serum it required twice as long to produce hemolysis in these cases. It can scarcely be asserted that the anemia is the causative factor, as the percentage of hemoglobin was not exceedingly low in any case. In one instance (the first experiment with No. 7) there was no reduction of hemoglobin, the percentage being the same as before inoculation.

TABLE VIII.  
*Titration to Test Hemolytic Power.*

Fowl No.	Date.	Titer.	Titer of normal control.
	1917		
3	Oct. 17	1:4	1:8
7	" 17	1:4	1:8
13	" 27	1:4	1:8

The findings are quite different in Table VII, which shows two cases (Nos. 6 and 17) of typical myeloid leucemia, and a mixed case (No. 8) characterized by a myeloid subleucemic blood picture and myelosis combined with intravascular leucostasis of the organs. In the mixed case there appears to be a slight retardation of hemolysis, but in the two cases of myeloid leucemia there is no diminution of hemolytic power. On the contrary, the hemolysis with serum from Fowl 17 is more rapid than that of the control. In view of these observations it is suggested that the power to produce hemolysis may depend upon the presence of myeloid tissue. This tissue, which was negligible in the first group of cases, was increased in the second group.

Although the experiments are few in number the phenomenon of a retardation in hemolysis appears to be a well established fact. As the determination of the moment of complete hemolysis depends to a certain extent on the personal element, as a matter of precaution titrations were done to test the hemolytic power in some cases in

<sup>3</sup> Agglutination of the erythrocytes invalidates the experiment.



Table VI. A series of dilutions (1:1, 1:2, 1:4...1:64) was made of active serum. After the components had been mixed as described above, they were kept at a temperature of 60°C. for 2½ hours and were shaken and placed in a cool room until the following day. The results are shown in Table VIII.

The titer, that is the dilution at which complete hemolysis is obtained, is shown to be in the case of the leucemic fowls about half that of the normal controls. This corresponds to the retardation of hemolysis which has been described.

### *Further Experiments.*

*Filtration of Inoculation Material.*—Previous experiments by Ellermann and Bang (1908, *a, b*, 1909) and Ellermann (1913, 1918) have proved that the virus is filterable, and that intact cells are not necessary in order to transmit the disease. As other investigators have had small success with positive filtration experiments, the results of an experiment with Strain H are given below.

15 gm. of the organs of Hen 1 (first experimental generation) were mixed with 0.9 per cent salt solution. This emulsion was filtered through cotton and diluted to make 100 cc. The fluid was passed through a Berkefeld filter No. 12. The filtrate was completely clear and gave no growth on agar or broth, while the control from the unfiltered emulsion gave rise to a rich growth. Ten normal birds (Nos. 7, 8, 18, and 40 to 46) were inoculated with the filtrate, each receiving 1 cc. Two fowls (Nos. 7 and 8) became infected. The incubation period and the total duration of the disease did not differ from those of the controls inoculated with unfiltered material.

*Immunization Experiments.*—Since experiments conducted by Ellermann and Bang and Hirschfeld and Jacoby have shown that subcutaneous inoculation does not infect fowls, it was decided to attempt immunization in this manner. Eight normal hens (Nos. 14 and 47 to 53) were inoculated subcutaneously with an emulsion of leucemic organs (No. 8), each hen receiving 2 cc. 3 weeks later the birds received an intravenous injection of emulsion from another infected hen (No. 5). One bird died 2½ months later. The postmortem and microscopic examinations showed a well marked leucosis. Hence it ap-

peared that the subcutaneous injection of virulent material had not produced immunity.

*Inoculation of Citrated Blood Plasma.*—These experiments were made in order to determine whether the virus is localized in the blood corpuscles or in the plasma or in both. 30 drops of blood were placed in a mixture of a 0.9 per cent salt solution and 10 per cent sodium citrate. After vigorous centrifugation the upper portion of the clear fluid was taken up in a sterile syringe and injected into eight normal hens (Nos. 15 and 54 to 60); eight other hens (Nos. 16 and 61 to 67) received injections of the blood cells. It was found that one fowl of each group (Nos. 15 and 16) contracted the disease.

In the experiments undertaken with another series of birds (Nos. 20 and 68 to 82) only one positive result was obtained with blood corpuscles, none with the plasma series. The objection might be raised that the separation of blood corpuscles and plasma cannot be considered effective, although this procedure is preferable to filtration, which injures the cells. The experiments seem to suggest that the virus is contained in the blood plasma. Nothing has been determined concerning the relation of the blood corpuscles to the virus. As it would hardly seem possible to remove a virus adherent to the surface of the cells, this has not been tried.

*Inoculation with Human Material.*—This experiment has no relation to the strain of fowl leucosis. It was undertaken solely to determine whether the disease in man and that in the fowl are caused by the same agent. Von Wiczowsky reports positive results in an experiment of this kind. On the other hand, experiments made by Hirschfeld and Jacoby (1909) and by Wechselmann and Hirschfeld were negative. *A priori*, the chances of a successful experiment seemed small, as attempts made to infect other birds of closely related species by means of fowl material have always failed. 2 cc. of blood taken from a patient, aged 43 years, suffering from myeloid leucemia in Bispebjerg Hospital, were mixed with a sodium citrate solution. 2 hours later this blood suspension was injected into eight normal hens (Nos. 83 to 90). The fowls were observed during a period of 6 months, but no symptoms of disease were discovered. Hence it seems doubtful whether the experiment of von Wiczowsky can be accepted. It is probable that every species has its own particular form of leucemia which cannot be transmitted to other species.

## SUMMARY.

1. A new strain of fowl leucosis has been transmitted through twelve generations of fowls.

2. An increase in virulence was observed during its passage. This was shown in a shortening of the interval between inoculation and death. The increase in virulence does not affect the number of successful inoculations, which remains approximately constant in from 20 to 40 per cent of the birds employed.

3. As with former strains, the disease manifests itself in various forms; *i.e.*, myeloid and intravascular lymphoid types. A single lymphatic case was observed.

4. In several intravascular cases a diminution in the hemolytic power of the serum was established. This phenomenon was absent in a number of myeloid cases.

5. Active immunization cannot be produced by means of the subcutaneous injection of virulent material.

6. The finding of previous experiments that the virus is filterable has been confirmed.

7. The inoculation of human leucemic material into fowls gave negative results.

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SUPERINFECTION IN EXPERIMENTAL SYPHILIS FOLLOWING THE ADMINISTRATION OF SUBCURATIVE DOSES OF ARSPHENAMINE OR NEOARSPHENAMINE.\*

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PLATES 73 AND 74.

(Received for publication, February 21, 1921.)

It is generally held that a person infected with the virus of syphilis becomes practically immune to a second infection—that, with the development of the initial lesion, a condition becomes established which makes it difficult or impossible to superimpose a second infection upon the one already present and that this refractory state is maintained as long as an infection exists. As far as they have been tested, human and animal experiences are in essential agreement upon these points.

In the absence of any evidence to the contrary, it has been assumed that the principles contained in this conception of syphilitic immunity apply to treated as well as to untreated cases of infection and many syphilographers have regarded the appearance of fresh lesions of the chancre type, under circumstances which would indicate a new infection, as the most conclusive evidence of the cure of the previous infection. There are some, however, who have questioned the validity of so called reinfections, and others who have objected to their interpretation as evidence of cure. Granting that the class of cases referred to represents instances of true, second infection, an alternative interpretation of such occurrences has recently been presented by Jacobi (1) in which it is suggested that the reaction to a second inoculation may be viewed more as an expression of an existing state of immunity than as evidence of the presence or absence of infection.

\* The results of the experiments reported in this paper were demonstrated at the All-America Conference on Venereal Diseases, Washington, D. C., December 6 to 11, 1920.

The situation which exists with reference to the immunity of infected individuals, the possibilities of superinfection or reinfection, and hence the interpretation to be placed upon the appearance of manifestations of disease which give every evidence of being due to a new infection are both complicated and obscure. Until the introduction of modern methods of spirocheticidal therapy, instances of so called reinfection were comparatively rare, and interest in the subject was largely a theoretical one. Within the past few years, however, the literature has contained numerous reports of reinfection following treatment with arsphenamine or neoarsphenamine, and the interpretation to be placed upon these occurrences has become a matter of immediate practical importance. Where the evidence of a new infection seemed sufficient, the general tendency has been to accept such infections as proof of a cure, and this would appear to be the logical interpretation unless it can be shown that under circumstances such as have existed in these cases, superinfection becomes possible. An element of uncertainty is introduced by the treatment employed, since practically nothing is known concerning the influence of such drugs as arsphenamine and neoarsphenamine upon syphilitic immunity.

The point at issue, therefore, is not so much a question of the immunity conferred by syphilitic infection as it is the effect which given therapeutic agents may have upon the resistance of infected individuals, whether native or acquired, and upon any spirochetes which may survive their action. Presented in this form, the problem of reinfection may be approached experimentally as one of the influence of drug action upon immunity and infection, and experiments have been carried out in animals for the purpose of determining the effect of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation. These experiments were divided into two groups according to the stage or progress of the infection, and the work to be reported in this paper deals with the results of treatment and reinoculation of animals with early but marked primary lesions.

#### EXPERIMENTAL.

The experiments to be reported consisted of the infection and treatment of two sets of rabbits—one with arsphenamine and the other with neoarsphenamine—after which they were reinoculated for the

purpose of determining their susceptibility to a new infection as indicated by the production of lesions at the site of inoculation. The results of the experiments were controlled in four ways, (1) as to the effects of the treatment employed, (2) as to the immunity developed by the infection, (3) as to the relative susceptibility of normal animals as indicated by their reaction to the virus used in the reinoculation experiments, and (4) by the use of a virus of essentially the same virulence as that causing the existing infection.

All animals were kept under observation for a minimum of 8 weeks after treatment while the therapeutic controls were held for 4 months as a means of affording a more accurate estimation of the status of the infection existing after treatment.

*Infection of the Animals Used.*—The animals used were inoculated in both testicles with 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field (Nichols strain). The object in view was the production of an intense infection which would confer a high degree of protection against reinoculation within a short period of time. The incubation period of the testicular lesions averaged about 10 days and the lesions developed very rapidly; at the end of 18 days the testicles were markedly enlarged and indurated, and in some instances there was an edema of the scrotum, indicating that the lesions were approaching the height of their first cycle of development. The animals were then divided into three groups according to the degree of development of the testicular lesions: Five animals with the most advanced infections were placed in the group to be treated and reinoculated; five others with the least advanced lesions were set aside as infected controls to be reinoculated at the same time as the treated animals; while a third group, consisting of animals showing various degrees of testicular involvement, were treated as therapeutic controls for the reinoculated animals of the first group.

*Treatment.*—Treatment was carried out 18 days after inoculation by the intravenous administration of a single dose of arsphenamine or neoarsphenamine. The products used were of the original German manufacture, being from Lots A 25819 and A 25884 respectively. The arsphenamine was neutralized by the addition of the theoretical amount of N sodium hydroxide to form the disodium salt, and both substances were administered in a 0.2 per cent solution.

The drugs were employed in the equivalent amounts as stated by the manufacturer. The doses used were 6 mg. of arsphenamine per kilo of body weight and 9 mg. of neoarsphenamine. The selection of these doses was based upon known values of therapeutic action for arsphenamine rather than neoarsphenamine and represented an attempt to use a dose of this drug which in the average animal of the group would yield a therapeutic effect of a definite character; namely, a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months. The ability to gauge these effects correctly was considered to be one of the two most essential features of the experiments, the other being reinoculation.

Twelve rabbits were treated with each of the drugs in the manner described; five of them were subsequently reinoculated and the seven others held as therapeutic controls.

*Reinoculation.*—Reinoculation of treated and untreated animals was carried out on the 24th day of the infection (5 days after treatment). In order that there might be as little difference as possible in the virulence of the organisms used for reinoculation and those producing the original infection, the virus used was obtained from an animal of the same series as those to be reinoculated. This was, in a sense, a measure of control. Each animal received an intracutaneous injection of 0.2 cc. of an emulsion containing one to three spirochetes to the microscopic field at two widely separated points, the ventral surface of the sheath and the base of the right ear in the region of the posterior marginal vein (see illustrations). It will be noted that one of these points was in close proximity to the original lesions and the other as far removed as was practicable. This choice of sites for reinoculation was determined in part by the susceptibility of skin areas and in part was used as a means of checking the extension of the immunity in control animals.

Reinoculations were timed both with reference to the possible retention of drug in a biologically active state and with reference to the progress of drug effects, the intention being to reinoculate as early as possible so as to give the best opportunity for the development of lesions before recurrence of the original lesions took place.

*Methods of Control.*—The results of the experiments outlined were controlled in four ways:



1. *Therapeutic Controls*.—In order to avoid any confusion which might arise from an attempt to interpret therapeutic effects obtained in animals which had been reinoculated, seven rabbits from each of the treated groups were held under observation for the purpose of determining as nearly as possible the effect which had been produced upon the original infection by the treatment employed.

2. *Infected Controls*.—Five infected rabbits of the same series as those used for treatment were reinoculated in the manner described with the same material as that used for reinoculation of the treated animals and for the inoculation of the normal controls.

3. *Normal Controls*.—The relative susceptibility of normal animals to the virus used for reinoculation was controlled by the inoculation of three normal rabbits carried out in the same manner as that of treated animals. These will be referred to as normal controls.

4. *Virus Control*.—As a means of insuring equality in the virulence of the spirochetes originally introduced and those used for the second inoculation, the reinoculations were made with material taken from a testicle of an animal of the same series as those to be reinoculated.

### *Effects of Treatment.*

A consideration of the results obtained from the experiments outlined above should logically begin with the effects of the treatment employed. Following administration of the drugs, the testicular lesions began to regress, resolution proceeding somewhat more rapidly in the animals treated with neoarsphenamine than in those with arsphenamine. In some animals, the lesions disappeared completely by the end of 7 to 14 days, at which time the effect of the drug upon existing lesions practically ceased.

Of the twelve animals treated with neoarsphenamine, the testicular lesions were completely resolved in seven, four showed definite residual lesions in the form of diffuse thickenings or of circumscribed nodules, and in one the result was uncertain. In contrast to this, complete resolution occurred in only four of the animals treated with arsphenamine—a difference which is quite characteristic of the action of the two drugs in inducing resolution or healing of lesions in the experimental animal.

The first evidences of relapse among the therapeutic controls were noted between 14 and 17 days after treatment. Four of the seven animals treated with arsphenamine showed reinduration and gradual increase in the size of residual lesions or the development of new foci of infection by the end of the 3rd week, and the infection progressed at a normal rate. A fifth animal relapsed 45 days after treatment, while the condition in the two others was not clear. From the 3rd week onward, there were brief periods during which the testicles of these two animals appeared to enlarge somewhat, there were ill defined areas of thickening, and even a few tiny nodules in the testicles or tunics, all of which were suggestive of relapse, but none of these conditions developed into affections which exhibited the characteristic clinical appearance of syphilitic lesions. At the end of 3 months, test inoculations were made from popliteal lymph nodes of both animals with positive results, and one of them developed a testicular lesion shortly afterwards.

Clinical relapse among the animals treated with neoarsphenamine was more delayed. Three animals of the control group showed minor changes suggestive of relapse as early as 14 days after treatment, but outspoken lesions did not develop until towards the end of the 3rd month. Relapse occurred in the four others between the 24th and 39th days, but in two of these the growth of the lesions was again rather slow and irregular for from 4 to 6 weeks.

Infection was, therefore, shown to be present in all the therapeutic controls of both series. In other words, none of the animals were cured by the treatment received, and similar but less marked effects were produced by the treatment given the animals which were used for reinoculation.

#### *Results from Reinoculation of Infected Controls.*

The second point to be considered is the probable state of immunity which had developed in the treated and reinoculated animals. It was not possible to determine this with certainty, and in reality such a determination was not essential to the object of these experiments. However, as the best means of estimating the immunity in these rabbits and of controlling the results of reinoculation at the same time,

which was of more importance, animals with the most advanced infections were selected for treatment, while those showing the least progress of the infection were used as controls. In this way, the state of the infection existing at the time of reinoculation of the untreated animals was practically the same as that which had existed at the time of treatment of the other group.

The results from reinoculation of the infected controls may be given briefly. Within a few hours, all animals of the series showed a slight acute reaction at the site of inoculation which consisted first of an edema and then of a slight diffuse redness about the site of inoculation. This reaction subsided completely within 24 to 48 hours and in one animal was the only reaction observed. In the others, a diffuse or papular infiltration developed in the sheath and at the base of the ear. The lesions in the sheath reached their height in from 5 to 7 days and disappeared completely within 12 to 14 days. The papules measured from 3 to 6 mm. in cross-diameter; they were of a rose-pink color, firmly indurated, and of a slightly translucent appearance. The more diffuse lesions presented essentially the same characteristics. The ear lesions developed somewhat more slowly, and in three of the five animals were very slight, diffuse infiltrations lasting approximately 3 weeks. The majority of the lesions described were of the type of slight non-specific inflammatory processes, or they might be regarded as allergic reactions. It is possible that some of them were due to a slight but transient local infection, but no examination was made for spirochetes for fear that the trauma inflicted might induce regression.<sup>1</sup>

In two instances out of the ten inoculations (two injections in each animal), small, firmly indurated, and translucent papules developed at the base of the ear (Figs. 1 and 2). Clinically, the lesions presented the appearance of syphilitic granulomata. One of them disappeared within 3 weeks, and while the other never developed to more than 4 to 6 mm. in diameter, it persisted for 54 days.

These were the only instances in which reinoculation resulted in the production of skin lesions which gave evidence of being due to

<sup>1</sup> While trauma in some form appears to play a part in the distribution and even in the development of syphilitic lesions in the rabbit, scarification, cutting, or aspiration with a needle frequently causes them to regress.

infection, and they are reproduced in Figs. 1 and 2 for purposes of comparison with the lesions obtained by reinoculation of the treated animals. It is to be noted that both lesions occurred on the ear, while the foreskin was entirely negative, which is the reverse of the order of susceptibility of the two skin areas in normal rabbits.

*Results from Reinoculation of Treated Animals and Normal Controls.*

Reinoculation of treated animals gave results which were strikingly different from those of the infected controls. In a word, all except two of them developed perfectly typical chancres, examples of which are given in Figs. 3 to 12; there was marked lymphadenitis such as is associated with primary lesions, and spirochetes were present in abundance. Within 7 days, every animal of the group showed a characteristic syphilitic reaction at the site of inoculation, either in the form of an elevated papule or of a flattened area of infiltration. Of the twenty points inoculated, nineteen were positive by the 7th day, and a lesion appeared at the one remaining focus on the 11th day after inoculation. The incubation periods of these lesions coincided with those of the normal controls.

The growth of both the sheath and ear lesions in four of the ten animals, including two treated with arsphenamine (Figs. 3 to 7) and two with neoarsphenamine (Figs. 8 to 12), was extremely rapid and practically uninterrupted until they reached the stages of development shown in Figs. 3 to 12, and some of them progressed beyond the points shown. On the other hand, none of the three normal controls developed lesions which were at all comparable to the sheath and ear lesions of these four treated animals.

In another animal (arsphenamine), the lesion on the sheath grew somewhat irregularly, but within 4 weeks formed a characteristic ulcerated chancre measuring 1 cm. in diameter. Growth then ceased for a short time, but the lesion was considerably larger and increasing actively when the animal was discarded. The ear lesion was of the nature of a papule surrounded by a zone of diffuse infiltration. It developed to approximately 8 mm. in diameter during the first 4 weeks but had practically disappeared before the animal was discarded.

The lesions of a sixth animal (neoarsphenamine) grew more rapidly than those of any other for about 3 weeks, but development ceased

at this point. On the sheath, there was a lenticular lesion measuring 5 to 7 mm. in thickness at its center and spreading diffusely over an area more than 1 cm. in diameter. A similar but less elevated lesion developed at the base of the ear, and while there was some exfoliation over the center of both lesions, neither of them underwent ulceration. They gradually subsided and had practically disappeared at the end of the 2 month period of observation. It is noteworthy that this animal developed a slight periostitis of the nasal bones 39 days after treatment and later a lesion of the cornea. There was also a marked popliteal lymphadenitis such as is commonly associated with focal infections in the drainage area.

In two other animals of the arsphenamine group, the reaction during the first few weeks after reinoculation was comparatively slight. During the 4th week, however, both the ear and sheath lesions of one animal began to increase rapidly and developed into characteristic chancre-like lesions of approximately 1 cm. in cross-diameter. The ear lesion of the other animal was first a papule, then a diffuse infiltration, but a typical chancre measuring 8 mm. in diameter developed on the sheath.

The lesions produced in the two remaining animals of the treated and reinoculated series were comparatively slight and consisted of small papules or diffuse infiltrations. They were more pronounced and more enduring than any lesion of the infected controls but less than those of the normal controls. The therapeutic response in one of these animals, both of which were treated with neoarsphenamine, was apparently less than that in any other animal of the series. The original lesions were never completely resolved, and a clinical relapse was recognized 17 days after treatment.

A feature of especial interest in these experiments was the fact that relapse of the original lesions occurred in eight of the ten reinoculated animals at about the time the superinfection lesions became well established. In most instances, the second chancres overgrew (inhibited) the recurrent lesions, but in other animals, both sets of lesions developed together.

An excellent illustration of this phenomenon of the double infection is furnished by Figs. 6 and 7. Inspection of Fig. 6 will show that there is a diffuse enlargement of the right testicle and two large nodules are well outlined at the positions indicated by the arrows. There is also a small patch of infiltration in

the left scrotum. The nodules in the testicle existed from the time of treatment, and although they were considerably softened and diminished in size, they became reindurated and began to enlarge by the end of the 2nd week. The development attained within 5 weeks (the time represented by this photograph) would of itself preclude the possibility of a metastatic origin. The further development of the two sets of lesions is shown in Fig. 7.

The results of the treatment and reinoculation experiments as a whole may be stated as follows: Of the five animals treated with arsphenamine, and then reinoculated, the original lesions were completely resolved in only one instance and relapse occurred within 33 days in four of the five animals, including the one animal whose lesions had been resolved. The other animal of the group showed no definite increase in the testicular lesions during the period of observation, and the lesions which resulted from reinoculation were less marked than those of the other animals. By reinoculation, characteristic chancres were produced in all the animals of the group. Three of the five animals developed well marked lesions at the base of the ear as well as on the sheath, while the ear lesions were comparatively slight in the two others.

The results after treatment with neoarsphenamine were not so uniform. The testicular lesions were quickly resolved in two of the five animals. The testicles of a third animal were left slightly enlarged and diffusely thickened, but no further change occurred, either in the way of regression or progression of the lesions during the period of observation, and while well marked lesions were produced by reinoculation on both the ear and the sheath, they were of shorter duration than those of the other animals. Clinical relapse occurred in all other animals of the group at from 14 to 24 days after treatment. Characteristic chancres were obtained from the second inoculation in three of the five animals on both the sheath and ear; the other two gave only diffuse or papular infiltrations.

The three normal controls developed characteristic chancres on the sheath which measured from 1 to 1.5 cm. in diameter. One of them developed a large nodule in the subcutaneous tissues at the base of the ear in addition to a diffuse infiltration of the skin and the usual lymphadenitis. In the two others, there were small papules surrounded by a zone of diffuse infiltration, but none of them developed chancre-like lesions in this location.

## DISCUSSION.

In attempting to give an interpretation of the experiments reported, the first point to be considered is that while one group of animals with well developed primary lesions of the testicles proved to be extremely refractory to a second cutaneous inoculation with a virus of equivalent virulence, a second group of animals from the same series, after treatment with arsphenamine or neoarsphenamine, was highly susceptible and with two exceptions reacted to the second inoculation with the development of characteristic manifestations of a primary infection. In fact, if susceptibility may be gauged by the reaction at the site of inoculation, some of the treated animals (four out of ten) were even more susceptible to infection than the normal controls. Disregarding for the moment the therapeutic result in as far as the original infection is concerned, it is certain that this difference in the reaction of the treated and untreated animals can be attributed to no other cause than the treatment employed.

In the second place, it is practically certain that none of the animals was cured. In the majority of them, the original lesions were not completely resolved, but there still might be some doubt as to whether the relapses which occurred in the reinoculated animals were true relapses of the original infection or were lesions arising from the second inoculation. In several instances, the clinical history of the relapse appeared to be sufficient in itself to exclude the latter possibility, since within a time too short to permit of the development of metastatic lesions, reinduration and growth occurred in existing lesions. In order to remove any possible confusion which might arise from this source, however, fourteen therapeutic controls were set aside—infected and treated with the same material and in the same manner as the reinoculated animals—and none of these was cured.

The results of the experiments may, therefore, be reduced to a very simple statement; namely, that treatment of animals with marked primary lesions of the testicles altered their resistance to such an extent as to render them susceptible to a second cutaneous infection without having effected a cure of the original infection. It is clear, therefore, that under the circumstances existing in these experiments, not only is superinfection of rabbits possible but animals treated in the manner

described may be rendered even more subject to a new infection than a normal animal.

It should be emphasized that these findings do not conflict in any way with established facts of syphilitic immunity. The conflict, if there is one, is with the assumption that the same conditions obtain in treated, as in untreated infections or that an immunity once established cannot be altered.

It has been clearly shown by the work of Finger and Landsteiner (2) and of others that immunity to a second infection is not always absolute—that by resorting to the use of large doses of virus, it is possible, even in advanced cases of syphilis, to produce superinfection, or lesions at the site of inoculation which tend to assume the form of those characteristic of the stage of infection during which reinoculation is carried out. It is also well known that superinfection with the production of typical chancres is comparatively easy during the first incubation period, and multiple chancres and autoinoculation with chancre-like lesions are explainable upon this basis.

The facts enumerated have been found to apply to both human and animal infections. From the data available, the chief differences which appear to exist in untreated infections are that protection against reinfection develops much more quickly, is more marked, and more enduring in the rabbit than in man. These few facts furnish all the basis necessary for an understanding of the phenomena of a second infection following treatment which is not curative. It is only necessary to consider that treatment with such substances as arsphenamine and neoarsphenamine may cause an infection to revert to the condition which existed during the first incubation period when spirochetes were present but no immunity had developed; further, that the spirochetes which survive the action of the drug employed may be so attenuated or enfeebled for the time being as to be incapable of arousing an antagonistic reaction on the part of the host, thus favoring the propagation of more vigorous organisms introduced from without and the production of characteristic primary lesions at the site of the new infection.

These were the postulates which formed the basis for the experiments reported, and the results would appear to justify the conclusion that, in as far as early infections of the rabbit are concerned,



treatment with arsphenamine or neoarsphenamine may alter the immunological status of the animal to such an extent as to favor the propagation of a second infection without having accomplished a cure of the first. In advanced infections, conditions are more complicated, and it is more difficult to obtain chancres from second inoculations, but experiments now in progress indicate that even here superinfection is still possible of attainment.

Apart from any bearing which these experiments may have upon problems of syphilitic immunity, they serve to emphasize the necessity for a careful consideration of the influence which any therapeutic agent or any system of therapy may exert upon the mechanism of animal resistance as well as the spirocheticidal action of the agents employed.

#### CONCLUSIONS.

From the facts presented, it may be concluded that the existence of an infection with *Spirocheta pallida* does not constitute a bar in itself to the introduction and propagation of a second infection in the same animal; that, just as there is a period following a first inoculation during which a second infection may be implanted with the production of characteristic primary lesions, conditions may again arise in animals which have once become refractory to a second inoculation, that will favor the introduction of a new infection with the formation of lesions presenting the characteristics of an original or first infection.

Experimentally, such a state may be induced in rabbits with early but well marked primary lesions of the testicles by treatment with either arsphenamine or neoarsphenamine, hence, treated but uncured animals may be rendered as susceptible to a second cutaneous inoculation as a normal animal, and the manifestations of disease resulting from the second infection may be indistinguishable from those of a first infection.

#### SUMMARY.

Experiments were carried out on rabbits for the purpose of determining the effects of subcurative doses of arsphenamine and of neoarsphenamine upon the resistance of infected animals to reinoculation with *Treponema pallidum* and hence the possibilities of the occurrence of a second infection in treated but uncured cases of infection.

All the animals used were inoculated with the same virus, and the experimental tests were carried out when the first cycle of testicular reaction was nearing its height. The animals with the most marked testicular lesions were used for the basic experiment of treatment and reinoculation. The results of this experiment were controlled from four different standpoints: (1) the effect of the treatment employed upon the existing infection; (2) the immunity present at the time of treatment; (3) the virulence of the organisms used for reinoculation as compared with those causing the existing infection; (4) the comparative susceptibility of normal animals to the virus used for reinoculation.

The results obtained showed (1) that the treatment employed was insufficient to cure any of the therapeutic controls; (2) that the infected controls were highly refractory to a second inoculation; (3) that the treated animals were highly susceptible to a second inoculation and although not cured of their original infection, reacted to the second inoculation with the formation of lesions indistinguishable from those of a first infection; (4) that in certain instances the treatment given had rendered infected animals more susceptible to infection than the normal controls.

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#### EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which represent the objects at their natural size.

#### PLATE 73.

FIGS. 1 and 2. Reinoculation lesions of control animals.

FIG. 1. A lenticular papule at the base of the ear 15 days after reinoculation. The lesion did not progress beyond the point shown.

FIG. 2. 30 days after reinoculation. There is an indurated papule in the same location. These were the most marked lesions produced by reinoculation of the infected controls.

FIGS. 3 to 7. Reinoculation lesions following treatment with arsphenamine.

FIGS. 3 and 4. Characteristic chancres on the ear and sheath of a treated animal as they appeared 42 and 37 days after reinoculation.

FIGS. 5 and 6. Ear and sheath lesions of an animal with clinical relapse of the original lesions—37 days after reinoculation. The right testicle (Fig. 6) is diffusely enlarged and indurated and contains two well defined nodules; the left testicle is still atrophic but there is a small area of infiltration in the scrotum.

FIG. 7. The genital lesions of the same animal 12 days later. Note the marked increase of all lesions and the extensive necrosis of the lesion on the sheath.

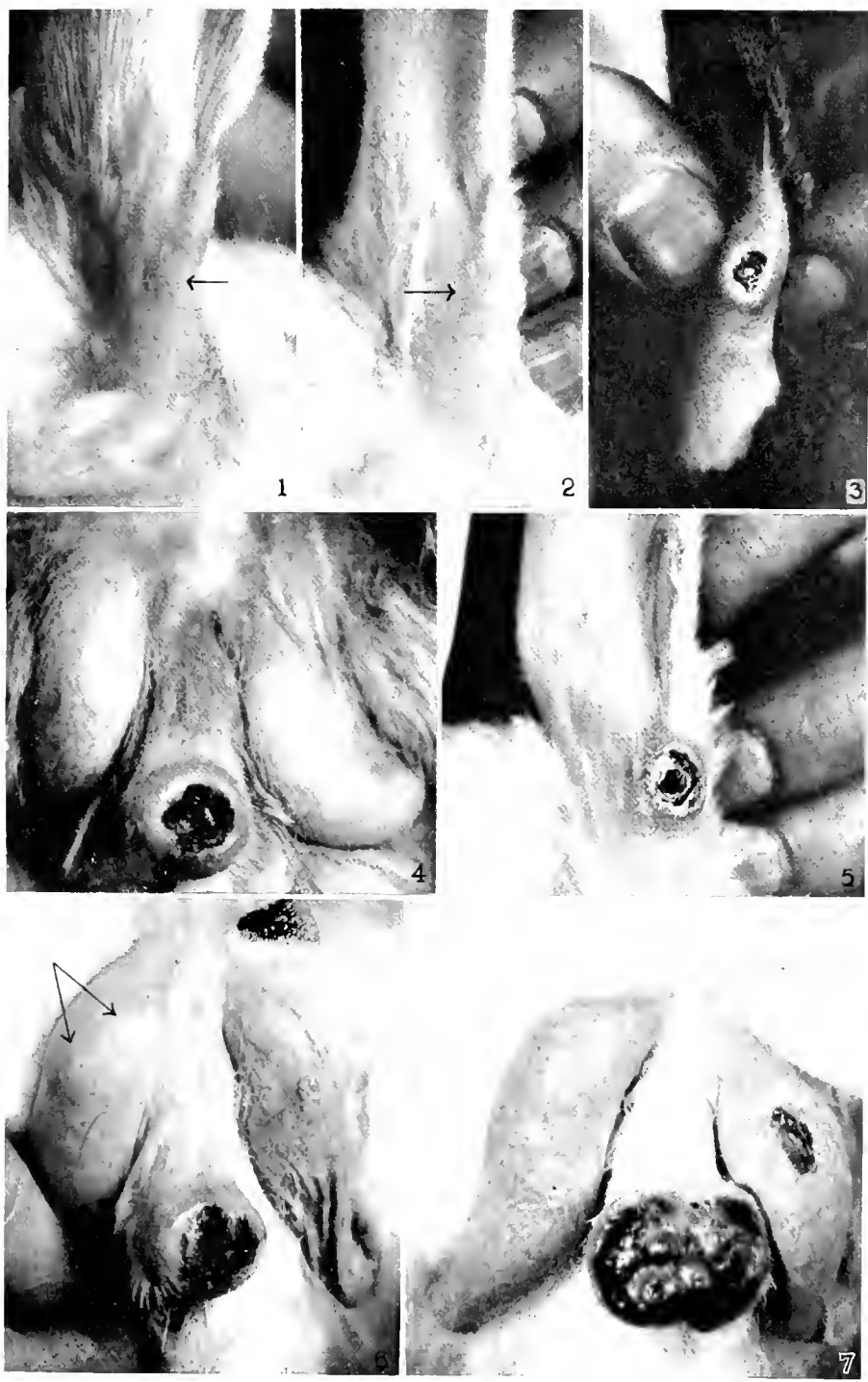
#### PLATE 74.

FIGS. 8 to 12. Reinoculation lesions following treatment with neoarsphenamine.

FIGS. 8 and 9. These figures are from the same animal and represent conditions as they existed 42 days after reinoculation. The two lesions are about equally developed and show marked necrosis.

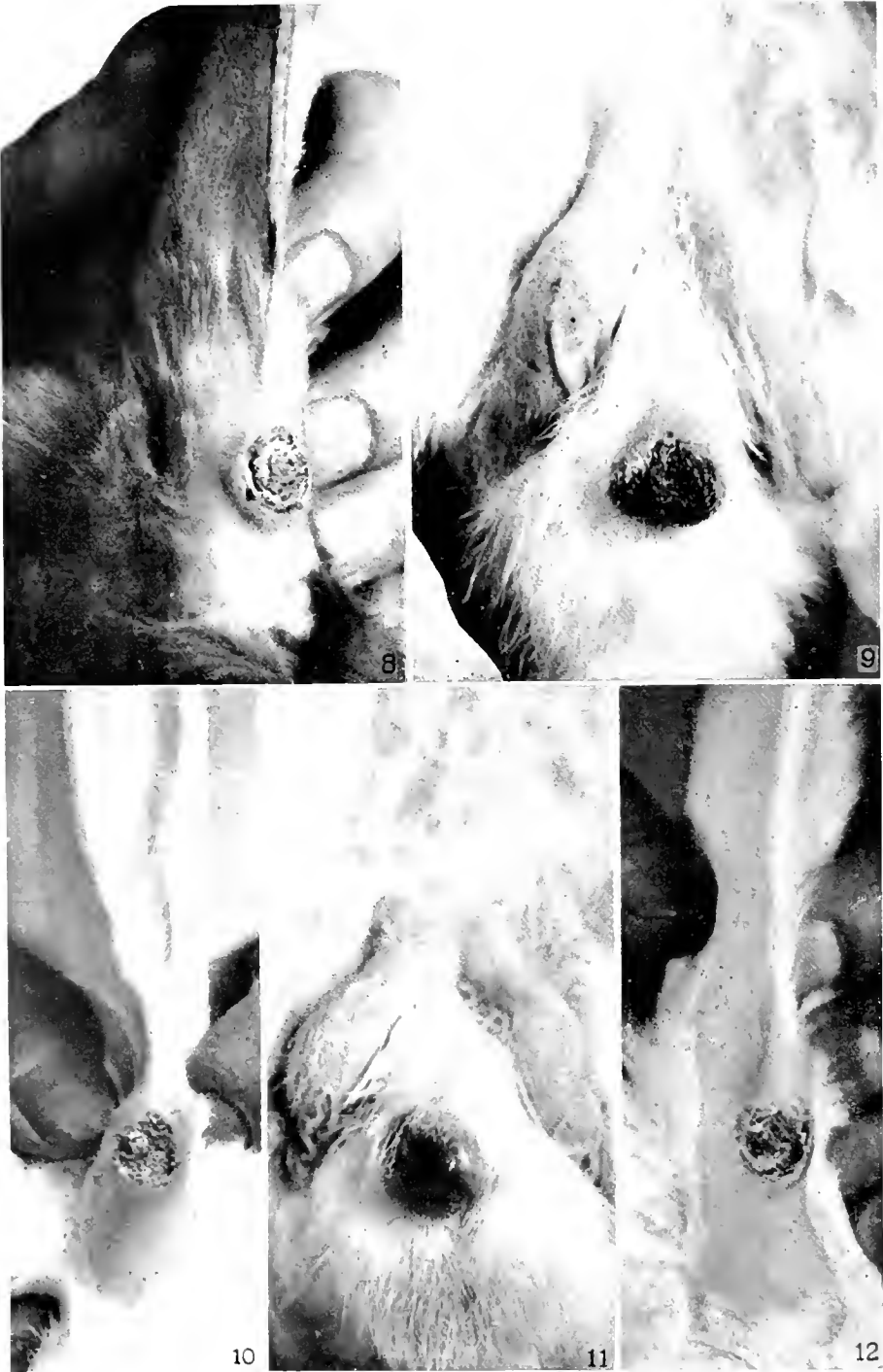
FIGS. 10 to 12. From a second animal. Figs. 10 and 11 are 42 days, and Fig. 12, 49 days after reinoculation.





(Brown and Pearce: Superinfection in experimental syphilis.)





(Brown and Pearce: Superinfection in experimental syphilis.)





## FURTHER STUDIES ON THE BEHAVIOR OF BACTERIA TOWARD GENTIAN VIOLET.\*

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PLATES 75 TO 77.

(Received for publication, November 3, 1920.)

### *Isolation of a Gentian-Positive Strain from a Culture of a Gentian-Negative Organism (a "Strain-within-a-Strain" Variant).*

When divided gentian violet plates are stroked with broth cultures or with heavy suspensions of *Bacillus coli*, growth is equally good on both sides of the plate (Fig. 1). So, too, if divided plates are poured so that each half receives a heavy inoculation of *Bacillus coli*, numerous colonies appear in both halves, though a similar experiment with *Bacillus subtilis* shows complete sterility of the gentian violet side. If, however, the inoculations are made with increasingly weak dilutions of the suspension, the colonies gradually become fewer on the gentian violet side and finally disappear altogether (Fig. 2). This phenomenon is exhibited whether a 2 or a 24 hour culture is studied. It may be demonstrated as well by a slightly different type of experiment. If a plain agar plate is stroked with broth cultures of *Bacillus coli* which have been exposed to the dye, the stained organisms appear to grow as well as the controls, though strokes of stained Gram-positive organisms, for example, *Bacillus subtilis* or *Oidium albicans*, will not grow at all (Fig. 3). If, however, the experiment is repeated with increasingly weak suspensions of *Bacillus coli* it will be seen that by no means all the organisms have actually survived exposure to the stain (Fig. 4).

\* A preliminary report of this work was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 17, 19, 20, 21).

It is therefore possible, by stroking divided plates with weak dilutions of *Bacillus coli* to obtain plates, with a few colonies on the plain agar side and none on the gentian violet agar. Some of these colonies may be presumed to have sprung from individuals which would have grown on the gentian violet agar if they had happened to be implanted there, others may be presumed to be made up of organisms incapable of growing in the presence of the dye. By again stroking divided plates with suspensions of these separate colonies an opportunity was afforded of isolating a strain of *Bacillus coli* incapable of growing in the presence of the dye, unmixed with individuals capable of such growth. This has, indeed, been done.

The result of this experiment was to provide two strains—labelled for convenience Strains X and Y—isolated from a single “pure” culture of *Bacillus coli*. These two strains, both definitely Gram-negative, are identical by all the usual tinctorial, morphological, and cultural tests. Yet Strain X grows well, Strain Y not at all, or practically not at all in the presence of gentian violet (Fig. 5); and strokes of Strain X which have been exposed to the dye and then planted on plain agar grow equally well with the controls, while strokes of Strain Y made with organisms which have been exposed to the dye do not grow at all (Fig. 6).

That this specific characteristic is not transient is shown by the experiment represented in Figs. 5 and 6. This experiment was done after the two strains had been allowed to grow for 6 weeks through a number of transplants, and it will be seen that the original features of the two strains have been retained.

The experiments outlined above demonstrate that within a single bacterial strain two strains may exist which, though identical in morphology and in tinctorial and cultural reactions, are dissimilar in their reactions to gentian violet, one growing vigorously and the other not at all on media containing the dye. These two types retained their differential characteristics after many transplantations. This isolation, from a pure culture, of a “strain-within-a-strain” variant parallels the observation reported some years ago of a “strain-within-a-species” variant occurring in the *Bacillus enteritidis* group.<sup>1</sup>

<sup>1</sup> Churchman, J. W., and Michael, W. H., *J. Exp. Med.*, 1912, xvi, 822.

The distinction between the two types of variant strain is, of course, not fundamental; one type was encountered in nature, the other was isolated in the laboratory.

*Explanation of the Parallelism between the Gram Reaction and the Gentian Violet Reaction.*

While certain facts in the observations hitherto reported on the parallelism between the Gram reaction and the gentian violet reaction were difficult to explain on the theory that the two reactions depended on a common factor and that the parallelism was therefore a fundamental one, the preponderance of evidence pointed in this direction. About 90 per cent of the Gram-positive organisms are killed by exposure to the dye and will not grow on media which contain it; about 90 per cent of the Gram-negative organisms grow well after exposure to the dye, and their growth seems to be unimpeded by the presence of the dye in media.

That this difference between the effect of gentian violet on the Gram-positive and the Gram-negative organisms might be applied to the isolation of Gram-negative bacteria from mixtures with Gram-positive bacteria, and particularly to the elimination of contaminations with *Bacillus subtilis*, which so frequently prove troublesome, was pointed out in a previous publication.<sup>2</sup> The lead thus given has since received the following practical applications: (1) isolation of *Bacillus tuberculosis* from sputum and feces (Petroff<sup>3</sup>); (2) elimination of false presumptive tests in the examination of milk and water for *Bacillus coli*, by the use of gentian violet (Hall and Ellefson<sup>4</sup>); (3) selective elimination of hay bacillus from cultures of obligative anaerobes by the use of gentian violet (Hall<sup>5</sup>); (4) isolation of *Bacillus influenzae* by the use of gentian violet (Bernstein and Loewe<sup>6</sup>); and (5) isolation of pathogenic moulds (*Epidermophyton inguinale*) by the use of gentian violet (Farley<sup>7</sup>).

<sup>2</sup> Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221.

<sup>3</sup> Petroff, S. A., *J. Exp. Med.*, 1915, xxi, 38.

<sup>4</sup> Hall, I. C., and Ellefson, L. J., *J. Bact.*, 1918, iii, 329, 355; *J. Am. Water Works Assn.*, 1919, vi, 67.

<sup>5</sup> Hall, I. C., *J. Am. Med. Assn.*, 1919, lxxii, 247.

<sup>6</sup> Bernstein, E. P., and Loewe, L., *J. Infect. Dis.*, 1919, xxiv, 78.

<sup>7</sup> Farley, D. L., *Arch. Dermatol. and Syphilol.*, 1920, ii, 466.

A parallelism so clear-cut and so amply confirmed could hardly be accidental, particularly as a similar parallelism was found to exist if the experiments were conducted with other dyes belonging to the triphenylmethane series. Moreover, it is just the constantly Gram-positive organisms like *Bacillus subtilis* which always fail to grow in the presence of even minute quantities of the dye, and just the constantly Gram-negative ones which grow readily in fairly strong dilutions of it (Fig. 1).<sup>8</sup>

The difference in avidity of the living organisms for the stain is also striking. *Bacillus subtilis* rapidly becomes deep violet and loses its motility; *Bacillus coli* or *Bacillus typhosus* stains slowly, only a small proportion of the individuals stain at all deeply, and all retain their motility for some time. If the Gram reaction is carried out with living organisms, centrifuged in a test-tube, the behavior of the negative and positive groups is the same as it is when they are killed and stained on a glass slide.

Facts of this kind led to the assumption that the parallelism between Gram reaction and gentian violet reaction depended on the presence in the Gram-positive organisms, and the absence from the Gram-negative ones, of unsaturated bodies avid for some portion of the triphenylmethane molecule. The saturation of these bodies would be thought of as accounting for the retention of the dye by the Gram-positive bacteria and their death in its presence; the absence of these bodies would explain why Gram-negative organisms fail to retain the dye and grow in media containing it.

There were, indeed, a few facts which would not conform to this hypothesis. There were in the first place the exceptional organisms; about 10 per cent of the Gram-positive strains studied were either unaffected by the dye or variable in their reactions to it, and an equal percentage of the Gram-negative cultures were somewhat susceptible to its bacteriostatic effects. Not only was this found to be the case in a study of all the common bacterial species, but an instance

<sup>8</sup> The chemical problems involved in the gentian violet reaction are now the subject of investigations in collaboration with Professor Bogert of Columbia University. It may be stated, however, that other members of the triphenylmethane series, which are themselves not dyes and possess relatively simple structural formulas, behave, as regards selective bacteriostasis, like gentian violet.

was encountered in which one strain of a species (*Bacillus enteritidis*)—and only one out of five studied—was quite sensitive to the bacteriostatic action of the dye, though the other four behaved quite according to rule, and all five were identical in cultural reactions and were definitely Gram-negative.<sup>9</sup>

An effort was made to test the fundamental nature of the parallelism between Gram and gentian reactions by training a Gram-positive and gentian-positive organism (*Bacillus subtilis*) to grow in the presence of the dye. If such an organism could be made in this way to lose its gentian positiveness without changing its Gram positiveness it would be clear that the two reactions did not depend on a common factor. If, on the other hand, both reactions changed, the opposite would be true.

The efforts to train *Bacillus subtilis* to grow in gentian violet were wholly unsuccessful. This organism will not grow on divided plates containing the dye in strengths of 1:1,000,000. It is possible, it is true, to induce it to grow in slightly stronger dilutions. But not much advance can be made, and the advance made is not retained; for the few organisms which have appeared in the stronger dilutions, if transplanted, fail to grow except in the weaker dilutions, around 1:1,000,000.

The problem was, however, solved in an entirely different way. If thick suspensions of the Gram-negative and gentian-negative *Bacillus coli* are stroked across a divided gentian violet plate, growth is equally vigorous on the two sides (Fig. 1). If, however, increasingly weak dilutions of the suspension are used for the stroking, the colonies on the gentian violet side of the plate become rapidly fewer (in proportion to those on the plain agar) as the dilution increases, and finally disappear altogether (Fig. 2). This is due, in part at least, to the fact, entirely obscured when thick suspensions are used for the experiment, that only a relatively small proportion of the

<sup>9</sup> It is interesting to note that Bronfenbrenner and his coworkers (Bronfenbrenner, J., Schlesinger, M. J., and Soletsky, D., *J. Bact.*, 1920, v, 24) in a study of the bacterial action of the CR indicator (China blue-rosolic acid) tested out this strain of *B. enteritidis* and found it to be exceptional also in its behavior toward CR. I have found that rosolic acid possesses selective properties similar to those of gentian violet.

individuals in a suspension of a Gram-negative organism are really gentian-negative; the majority will not grow in the presence of the dye.<sup>10</sup> If the suspension used for stroking the plate is thick, this small proportion of gentian-negative individuals is (absolutely) sufficiently large in quantity to produce good growth in the presence of the dye. If, on the other hand, the suspension is weak the gentian-negative individuals are, not only relatively, but absolutely few in number; few colonies therefore appear on the violet agar; if the dilution is very weak, none appears.

It is not difficult to isolate in pure culture the individuals which will and those which will not grow in the presence of the dye (Figs. 5 and 6). When so isolated the gentian-positive strain, that is the strain which will not grow in the presence of the dye, is found to be as definitely Gram-negative as the gentian-negative strain. The factor which determines the reaction of an organism to the Gram process of staining is not therefore the same as the factor which determines its growth reaction in the presence of gentian violet.

*Effect of Repeated Reinoculations of Gentian Violet Agar with a Gentian-Positive Organism.*

If a divided gentian violet plate containing the dye in a dilution of 1:100,000 is stroked with a thick suspension of the Gram-positive and gentian-positive *Bacillus subtilis*, no growth will ever occur on the gentian violet side of the plate; the organism will, indeed, fail to grow up to the dividing line between plain and gentian violet agar, ceasing sharply at a point 0.5 to 1 cm. from this line (Fig. 1). This inhibition takes place even when the dye is present in extremely weak dilutions. Growth is prevented by dilutions of 1:1,000,000 (Fig. 7), inhibited by 1:2,000,000, and only becomes vigorous at a strength of 1:3,000,000.

If, however, the gentian violet half of the plate is repeatedly and heavily reinoculated on successive days in the same place, a fair growth,—in some instances a rather vigorous growth—may finally

<sup>10</sup> It cannot be said, since the whole bacterial field has not been studied with this point in view, that all Gram-negative species contain these two strains. The specimen of *B. coli* with which the experiments were done and the specimen of *B. typhosus* and *B. prodigiosus* examined contained them.

be obtained. In Fig. 8 such an experiment is shown. Two strokes of a thick suspension of *Bacillus subtilis* were made on this plate. Nothing further was done to the right-hand stroke and the usual effect of the dye is clearly shown. In the left-hand stroke from the edge where the original growth on the plain agar ceased (this edge will be readily recognized), organisms were repeatedly resmeared over the gentian violet agar; a fair growth resulted.

That this growth which results from restroking is not due to acclimatization of organisms to the dye seems likely from the fact that prolonged attempts at experimental acclimatization of *Bacillus subtilis* to gentian violet have been unsuccessful. Furthermore, positive proof that acclimatization is not the explanation is given by the fact that if organisms which have, by reinoculation, been thus induced to grow on gentian violet agar, are freshly smeared on another divided plate they will not grow on the side of the plate which contains the dye. This phenomenon can hardly be due to insufficiency of dye in the presence of increase of inoculated organisms, for in the plates used in the experiments dye was present in strengths (1:100,000) greatly in excess of the strength necessary to prevent the growth of *Bacillus subtilis* (1:1,000,000); and owing to the great diffusibility of the dye through agar much of that present in the plates is available for the organisms.

Whether the phenomenon is due to a change in the dye caused by organisms which, though not surviving, live sufficiently long to prepare the soil for subsequent implants; whether it is due to some protective barrier or nutritional or growth-accelerating substance provided by the dead bodies of bacteria to the living bacteria which rest upon them in subsequent seedings; or whether it is due to a communal activity of organisms—to which further reference is made in the following paper—are questions at present under experimental study. But the fact itself is sufficiently significant for certain problems in the chemotherapy of infections.

In searching for a possible explanation for these facts it is important that attention should be called to experiments which demonstrate the effect of the presence of dead bacterial bodies in media on which transplants are made. An experiment of this sort is illustrated in Figs. 9 and 10. The tubes contained gentian violet agar.

The surface of the control tube (Fig. 9) was inoculated with *Bacillus subtilis* and, as this organism never grows in the presence of the dye, no growth occurred. The surface of the other tube (Fig. 10) was covered with a thin layer of killed, washed organisms<sup>11</sup> (*Bacillus subtilis*). After this layer dried an inoculation of living *Bacillus subtilis* was made on top of it. Growth occurred, owing apparently to a complete protection of some sort interposed by the dead bodies between the living bacteria and the dye, or to some effect of the dead bacteria on bacterial growth. A similar result is obtained if dead bodies of *Micrococcus aureus* or of *Bacillus coli* are smeared on the surface of the gentian violet agar instead of dead bodies of *Bacillus subtilis*. Such an experiment, besides bringing out a fact to which attention has not hitherto been directed, shows clearly that dead bacterial bodies lying in a wound may entirely alter the reaction between a bacteriostatic agent and the living organisms which one is attempting to reach.

*Selective Activity of Gentian Violet in Relation to Chemotherapy.*

That the selective bacteriostatic action of gentian violet, demonstrable *in vitro*, is capable of clinical application in the treatment of infections has been amply demonstrated by experiments conducted in the hospital wards during the last 6 years. It has been shown that in certain types of acute infections of joints, staining of the synovial membrane (after proper mechanical cleansing by special methods) leads to prompt sterilization and clinical cure.<sup>12</sup> It has also been shown that with this dye it is possible to rid granulating wounds of

<sup>11</sup> The organisms were grown on medium containing only distilled water and agar, in order that their bodies might contain the minimum amount of nutritive substance. These bacterial bodies were washed six times with distilled water and were killed. Suitable controls were made to insure sterility. In order to prove that no available food material was still present, a control experiment was done in which these dead bacterial bodies were smeared on the bottom of a Petri dish, dried, and then inoculated with live *B. subtilis*, suitable moisture being provided. No growth occurred on incubation. The growth of *B. subtilis* in the experimental tubes illustrated in Fig. 10 was not, therefore, due to any nutritive material carried over in the dead bacterial bodies.

<sup>12</sup> Churchman, J. W., *J. Am. Med. Assn.*, 1918, lxx, 1047; 1919, lxxii, 1280; 1920, lxxv, 583.



certain organisms which have resisted other bacteriostatic agents; this was proved by the successful treatment of two patients with thigh amputations at the Walter Reed Hospital. The stumps of these individuals had become diphtheria carriers, but by using the dye they were rid of organisms which had persisted for weeks in spite of all treatment.<sup>13</sup> That the selective power of the dye, so clearly shown in divided plates, would also be demonstrated by a study of its effects in wounds was well shown during these efforts to sterilize infected amputation wounds. *Bacillus diphtheriae* was caused to disappear without much difficulty, but in a stump infected with *Bacillus coli* it was impossible to rid the wound entirely of its organisms.

In spite of this considerable amount of clinical success in the treatment of a certain type of infection with gentian violet, the observations reported in the previous communications indicate that selective bacteriostasis is a very complex process and point out the difficulties attending the direct transfer of laboratory findings into therapeutics. The method of divided plates presents an excellent means of studying the interaction between dye and organism; the isolation of a pure strain of gentian-negative *Bacillus coli* makes it possible to investigate the question by single cell experiments of a peculiarly reliable kind; and the selective property of gentian violet may be taken advantage of for the study of the various elements of selective bacteriostasis on a single plate. Such a plate is represented in Fig. 11. This experiment was done to demonstrate the futility of generalizations about the effect of a chemotherapeutic agent on bacteria, even when the generalization is confined to its effect *in vitro*, and to indicate the necessity of bearing in mind, when drawing conclusions from experiments in this field, the conditions under which the experiment was done. On this plate, as will be seen (Fig. 11) seven different effects of gentian-violet on bacteria are shown, the result obtained depending on the organism chosen for the experiment and the conditions under which the inoculation was made. These different results are as follows: (1) Thick suspensions of Gram-positive organisms (*Bacillus subtilis*) will not grow in the presence of the dye (Fig. 11, A). (2) By repeated reinoculations of these organisms a moderate

<sup>13</sup> Churchman, J. W., *J. Am. Med. Assn.*, 1920, lxxiv, 145.

growth can be procured on the gentian violet agar (Fig. 11, *B*). (3) Thick suspensions of the Gram-negative *Bacillus coli* grow equally well on the two halves of the plate (Fig. 11, *C*). (4) If weak dilutions of a suspension of *Bacillus coli* are stroked across the plate almost no growth occurs on the gentian violet side (Fig. 11, *D*). (5) A thick suspension of the gentian-negative strain of *Bacillus coli* grows equally well on the two halves of the plate (Fig. 11, *E*). (6) If a weak dilution of the gentian-negative strain of *Bacillus coli* is stroked across the plate no growth may occur on the gentian violet side (Fig. 11, *F*). (7) If a thick suspension of the gentian-positive strain of *Bacillus coli* is stroked no growth occurs on the gentian violet agar (Fig. 11, *G*).

Unless considerations of this kind are borne in mind one may easily fall into gross error. For example, if the original experiments had been conducted with a thin suspension of *Bacillus coli* and the result seen at *D*, Fig. 11, had been obtained the conclusion would have been drawn that gentian violet has a strong bacteriostatic effect on *Bacillus coli*; whereas if thick suspensions are used, the opposite proves to be the case (Fig. 11, *C*). This error has, as a matter of fact, been committed by certain observers working with other dyes.

Since the single cell method of Barber<sup>14</sup> offers an important method for the study of reactions between chemical substances and single bacteria, it cannot be too strongly emphasized that the effect of a bacteriostatic agent on a group of bacteria may be quite different from its effect on individual organisms.

That the effect of a chemotherapeutic agent should be conceived in terms of stasis rather than death has been proved by the whole trend of the work with gentian violet. The difficulty of proving that organisms which have apparently been killed by chemical agents are really dead may be greater than might at first be supposed; and in any experiments in this field it is well to remember that organisms apparently killed may survive and give signs of life after a long period of lag. A striking instance of this phenomenon is shown in Text-fig. 1. This experiment was one of a series done to determine by animal inoculations whether stained gentian-positive organisms, which do

<sup>14</sup> Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

not grow in culture media, are actually killed by exposure to the dye. Two series of animal inoculations—one with *Staphylococcus aureus* and the other with a strain of *Blastomyces* pathogenic for guinea pigs—seemed to warrant the conclusion that the stained organisms were killed, for all the animals that received unstained organisms died of the disease, and all those that received stained organisms survived. With stained *Bacillus anthracis*, however, different and striking results were obtained. This organism will not grow when it is planted on agar after having been stained. When injected in large amounts,

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
M.1	—	x																										
M.2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	x	
M.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
R.1	—	—	—	—	—	x																						
R.2	---	---	---	---	---	---	x																					
R.3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
R.4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

TEXT-FIG. 1. Results of animal inoculation with stained *B. anthracis*. M., mouse; R., rabbit. The course of control experiments is represented by a solid line, the course of experimental animals by a broken line. X indicates that cultures made at death from the heart's blood and spleen were positive for *B. anthracis*. Mouse 3 and Rabbits 3 and 4 survived without symptoms. Mouse 2 died of anthrax on the 26th day; the blood and spleen swarmed with the organisms.

after staining, into the susceptible mouse, it often causes death, though not infrequently the inoculated animal lives. But the point worthy of note is that when the animal dies it is sometimes after a period of many days of perfect health, though the control mice die in 36 hours. Mouse 2, for example (Text-fig. 1) died of anthrax on the 26th day, having been in perfect health in the interval; that is, it behaved as if it had received the injection more than 3 weeks after the inoculation had actually been made. The stained organisms, nearly but not quite killed by their exposure to the dye, had suffi-

ciently recuperated at the end of 26 days to bring about the death of the animal from anthrax. In the animals which survived the inoculation entirely it is not unlikely that the defensive mechanism of the body was successful in overcoming the bacteria, which were injured but not killed. The experiments prove that the survival of injected animals does not necessarily prove that the organisms injected were dead.

#### SUMMARY.

1. A gentian-positive strain (a "strain-within-a-strain" variant) has been isolated from a pure culture of a gentian-negative organism. This observation corresponds to that of a "strain-within-a-species" variant, occurring in the *enteritidis* group, reported some years ago.

2. The Gram reaction and the gentian reaction do not depend, as has been assumed in previous publications, on the specific affinity of the gentian-positive organisms for a portion of the gentian violet molecule, since certain Gram-negative strains are shown to be gentian-positive.

3. Dead bacterial bodies interposed between living bacteria and gentian violet media partially negative the effect of the dye on Gram-positive organisms and allow them to grow. This seems to be either a phenomenon of filtration or of stimulation of growth.

4. The application of these facts by the method of divided plates shows a number of difficulties in the application of laboratory studies to chemotherapeutics, which would escape observation by ordinary methods.

#### EXPLANATION OF PLATES.

##### PLATE 75.

FIG. 1. Divided gentian violet plate (gentian violet 1:100,000). *T*, *B. typhosus*; *S*, *Micrococcus aureus*; *O.A*, *Oidium albicans*; *Su*, *B. subtilis*; *C*, *B. coli*. It is the strongly Gram-positive organisms that fail to grow and the strongly Gram-negative ones that grow.

FIG. 2. 24 hour cultures of *B. coli*. Disappearance of all colonies from the gentian violet agar side of the plate as the dilution of suspension used for stroking becomes weaker. *C*, *B. coli*; *Broth*, broth culture (undiluted).

## PLATE 76.

FIG. 3. Effect of staining on Gram-negative *B. coli* and Gram-positive *Oidium albicans* (cf. Fig. 4). *C*, unstained *B. coli* (control); *C'*, stained *B. coli*; *O*, unstained *Oidium albicans* (control); *O'*, stained *Oidium albicans*.

FIG. 4. Effect of staining increasingly weak dilutions of a suspension of *B. coli* with gentian violet. *Con*, unstained organisms; *St*, stained organisms.

FIG. 5. Behavior of Strains X and Y on a divided plate 6 weeks after first isolation.

FIG. 6. Behavior of Strains X and Y on exposure to gentian violet 6 weeks after first isolation. Plain agar plate. *X*, unstained organisms (control); *X'*, stained organisms. *Y*, unstained organisms (control); *Y'*, stained organisms.

FIG. 7. *B. subtilis* on a divided plate. The upper half contains gentian violet in 1:1,000,000 dilution. (*A* refers to the series of experiments.)

FIG. 8. Two strokes of a thick suspension of *B. subtilis* were made. On the right nothing further was done. On the left reinoculations were made on the gentian violet agar.

## PLATE 77.

FIG. 9. Implantation of *B. subtilis* on the surface of gentian violet agar; no growth resulted. *a*, site of inoculation.

FIG. 10. Implantation of *B. subtilis* on gentian violet agar the surface of which had been covered with a thin layer of killed, washed organisms. *a*, colony of *B. subtilis*; *b*, layer of killed washed organisms (*B. subtilis*); *c*, photograph of the surface of the tube, showing the colony of *B. subtilis*.

FIG. 11. The figure shows how the result of an experiment in glass on the effect of a bacteriostatic agent may vary with slightly changing conditions and illustrates some of the difficulties encountered. *A*, stroke of a suspension of *B. subtilis*; *B*, stroke of a suspension of *B. subtilis* with reinoculation of gentian violet agar; *C*, stroke of a thick suspension of *B. coli*; *D*, stroke of a thin suspension of *B. coli*; *E*, stroke of a thick suspension of Strain X; *F*, stroke of a thin suspension of Strain X; *G*, stroke of a thick suspension of Strain Y.





FIG. 1.



FIG. 2, a.



FIG. 2, b.



FIG. 2, c.

(Churchman: Behavior of bacteria toward gentian violet.)





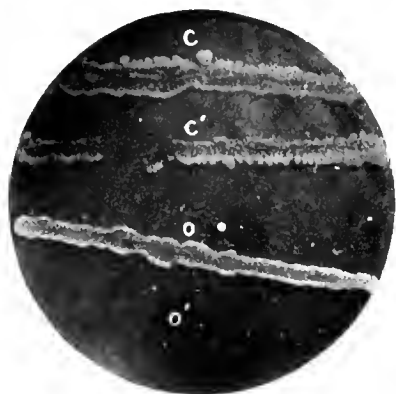


FIG. 3.

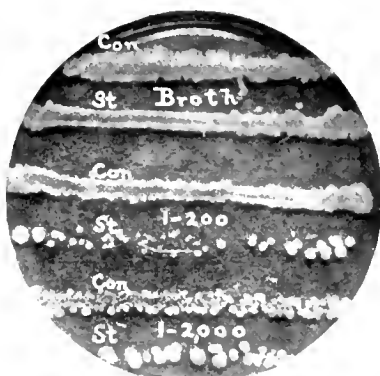


FIG. 4.



FIG. 5.

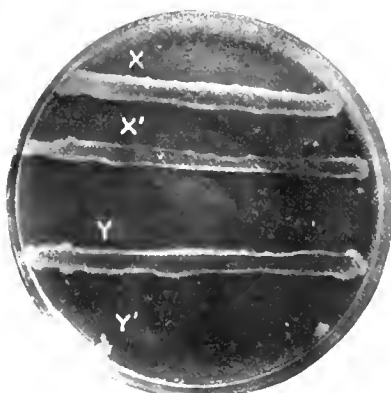


FIG. 6.



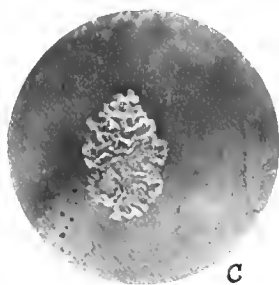
FIG. 7.



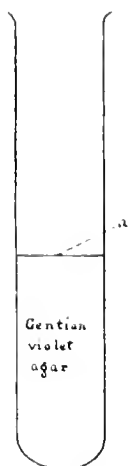
FIG. 8.

(Churchman: Behavior of bacteria toward gentian violet.)





C



CONTROL

FIG. 9.

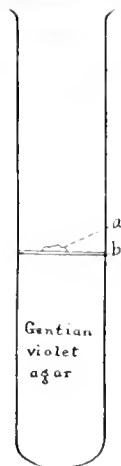


FIG. 10.

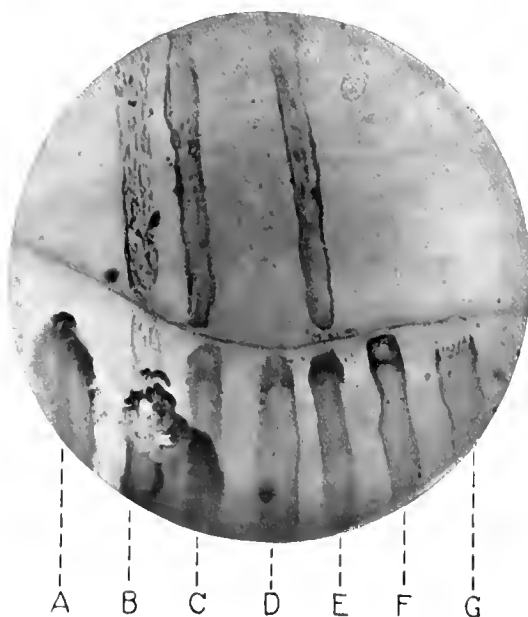


FIG. 11.

(Churchman: Behavior of bacteria toward gentian violet.)



## COMMUNAL ACTIVITY OF BACTERIA.\*

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PLATES 78 TO 80.

(Received for publication, November 3, 1920.)

The isolation of a strain of *Bacillus coli* fast to gentian violet, that is one containing no individuals susceptible to the bacteriostatic properties of the dye,<sup>1</sup> has made it possible to study quantitatively the reaction between this bacteriostatic agent and bacteria, without encountering the disturbing factor usually met in such studies which results from the variability in susceptibility of individual organisms to the chemical substance under examination. This strain—Strain X—had been isolated from a single colony growing on gentian violet agar, and had been kept growing, by frequent transplants, on media containing the dye, over a period of several weeks.<sup>2</sup> The ability of every individual to grow in the presence of the dye had therefore been proved.

With this strain a large number of single cell and small group transplants have been made in order to see whether any difference could be observed between the behavior of isolated individual organisms and that of very small aggregations of the same organisms. The experiments showed that a marked difference exists.

The technique used was that of Barber.<sup>3</sup> Only motile organisms were transplanted. The transplants were always made with young broth cultures 2 to 4 hours old, but it was found equally important to use for the stock cultures, from which the broth cultures for study were made, fresh agar transplants not more than 18 hours old; when

\* A preliminary report of these observations was published in the *Proceedings of the Society of Experimental Biology and Medicine* (Churchman, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 22).

<sup>1</sup> Churchman, J. W., *J. Exp. Med.*, 1921, xxxiii, 569.

<sup>2</sup> See Figs. 5 and 6, of the preceding paper.<sup>1</sup>

<sup>3</sup> Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

older agar transplants were used there were delay and inconstant results in the controls. We observed this type of lag, due to the age of the culture from which the subculture for the transplantation was made, independently, before knowing of Chesney's work.<sup>4</sup> To bear it in mind in single cell work is essential.

To determine the behavior of single cells in the presence of gentian violet, transplantations were made onto gentian violet agar and into gentian violet broth, and by way of comparison, similar transplants of small groups of cells were made into the same media.

#### *Transplantation onto Gentian Violet Agar.*

Although, as has been said, the organism with which the experiments were done was definitely gentian-negative and grew with apparently no inhibition when strokes of a heavy suspension were made onto agar containing the dye,<sup>5</sup> transplants of single cells almost never grew, although as high as 85 per cent positives were obtained in the controls. In the only two instances in the whole series of single cell transplants in which growth occurred, marked delay took place, a delay which was never observed in the controls. Moreover, transplants of small groups of organisms (five to fifteen) did not grow, though transplants of thirty individuals grew regularly (Figs. 1 to 5).

#### *Transplantation into Gentian Violet Broth.*

The experiments just cited made it seem likely that there was some fundamental difference between the behavior of a single cell and that of a small group of cells in the presence of gentian violet and this probability became a certainty as a result of the transplantations made into gentian violet broth in a dilution of 1:100,000, pH 7.2. The gentian negativeness of the organism used for this series of experiments was even more authentic than that of the one used in the agar experiments. It came from the colony marked *B* in Fig. 5. This was one of the only two colonies which ever appeared in our experiments after transplantation of a single cell onto gentian violet agar. Colony *A* on this plate was just visible at the end of 18 hours, though

<sup>4</sup> Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

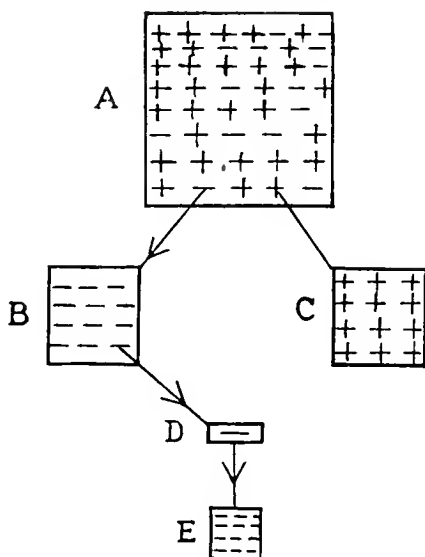
<sup>5</sup> See Fig. 5 of the preceding paper.<sup>1</sup>

the controls were well developed at this time (Fig. 3). At the site where Colony B subsequently developed nothing was at this time to be seen, even with a magnifying glass; Colony B appeared during the second 24 hours.

The organisms in Colony B provided material for a study of the effect of gentian violet on single cell transplants that was above criticism. Not only had it come from a pure gentian-negative strain and been kept growing for weeks on gentian violet media, but the organisms in Colony B were all the descendants of a single cell which had survived and reproduced in the presence of the dye. One could feel certain that every individual in this culture was gentian violet-negative; one did not have to consider the possibility that failure of growth in single cell transplants might be a matter of chance and due to the presence of individuals susceptible to the dye, which happened to be picked up. As a matter of fact, this possibility of a chance picking up of susceptible organisms could hardly be seriously considered as an explanation of the results, if the large number of transplants made is borne in mind.

The pedigree of Colony B is shown in Text-fig. 1. The results of the inoculation of broth with this strain which was called Strain Z showed beyond doubt that there is a fundamental difference between the behavior of one cell and that of a small group of cells toward gentian violet. 80 per cent positives were obtained in the controls in which single cells were inoculated into plain broth; and almost 100 per cent positives were obtained when thirty or more cells were planted in gentian violet broth. Yet when single cells were planted in gentian violet broth, or when very small groups (two to eight organisms) were seeded, growth did not occur (Figs. 6 and 7). On the basis of 140 successive single cell transplants into gentian violet broth without growth in any instance, the conclusion seemed justified that single cells would never grow under these conditions. In a final series, however, of eight inoculations delayed growth was obtained in one tube in the second 24 hours, an occurrence so rare that it seems justifiable to regard it as the occasional and unexplained exception met with in the study of almost every biological phenomenon. These negative results with single cells were striking when compared with the almost absolute constancy with which growth occurred when groups of thirty or more cells were inoculated.

The term communal activity of bacteria is used to express the facts just detailed. This term is open to the objection that bacterial interreactions are implied, for which there is at present no rigorous evidence. It might be that thirty cells succeed in growing, merely because thirty cells are able to produce some antidy substance in an amount sufficient to destroy the bacteriostatic effect of the dye, while one cell fails to grow because it is unable to do this. Without



TEXT-FIG. 1. Pedigree of Strain Z. *A*, original culture of *B. coli*, containing gentian-negative and gentian-positive individuals; *B*, pure culture of gentian-negative strain, isolated from *A*; *C*, pure culture of gentian-positive strain isolated from *A*; *D*, single cell from Strain *B*; *E*, colony resulting from planting Strain *D* on agar.

implying anything as to the underlying source of the phenomenon we use the term communal activity to describe it in order to indicate that in the contest between bacteria and bacteriostatic agents very small groups of organisms may be able to accomplish together what they could not accomplish if working singly. The significance of these facts for the study of chemotherapy and particularly for the study of the effect of bacteriostatic agents by the single cell method is clear. One cannot conclude from the behavior of a single cell in the



presence of such a substance anything as to the behavior of a group of cells, even a very small group, under the same conditions.

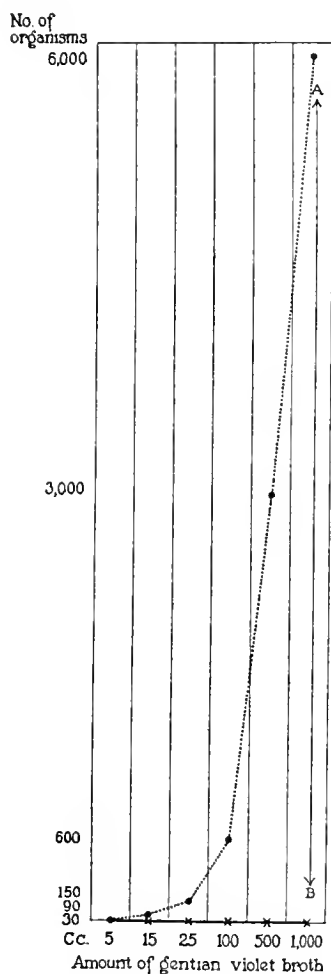
It may perhaps be again emphasized that the results obtained in these experiments were clearly not due to individual variants, susceptible to gentian violet, which happened to be picked up in making the single cell transplants. Not only was the strain used one which had proved its insusceptibility to gentian violet by growing in the presence of this dye, but the failure of growth in 140 consecutive single cell transplants into gentian violet broth eliminates, by the law of probabilities, such an explanation.

It should be clear that the fact here established is of a different order from the well known relation between growth and gross size of inoculum. The presence of large numbers of dead organisms in the ordinary culture and of organisms which, though living, are susceptible to the slightly unfavorable conditions of the new media into which they are transplanted makes it more probable that growth will follow the inoculation of media with 500,000,000 organisms than if only 500 are used. But in the experiments described here only living, motile organisms were used, and only individuals from a strain which had proved its ability to grow in the presence of the dye and did so constantly when inoculated in groups of thirty or more. It seems probable that some factor not hitherto recognized must be found to account for the difference in behavior between one cell and thirty cells and that it is a factor other than that which accounts for the difference in behavior between 500 cells and 500,000,000.

We have done a large number of experiments to determine whether the facts just detailed were to be explained simply by the relation of the number of transplanted organisms to the amount of available gentian violet; that is, whether the whole phenomenon is a purely quantitative one. Large inoculations of this gentian-negative strain grow in the presence of gentian violet without any apparent restraint; so, too, do inoculations of thirty cells; whereas single cells, under identical conditions, do not grow at all. This might be due to the fact that groups of cells, even small groups of thirty individuals, were able to make some change in the dye, gentian violet being assumed to offer a slightly unfavorable medium even for this gentian-negative strain, in spite of the absence of any apparent inhibition to the growth

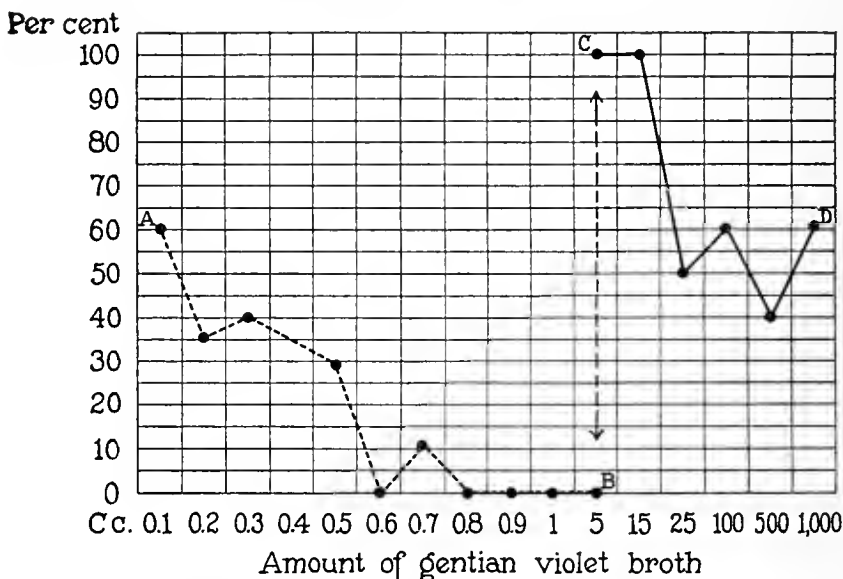
of groups of cells. Single cells might be unable to effect this change in dye in an amount sufficient to allow growth to take place. If this explanation were the correct one, it should be possible to demonstrate an approximate relation between the number of organisms and the amount of gentian violet broth in which a given group could grow. In the experiments thus far described the transplants were made into 5 cc. of 1:100,000 gentian violet broth. Under these conditions one cell would not grow but thirty cells would. If it is assumed that this was because thirty cells could produce thirty times the amount of antidye substance produced by one cell, then by merely multiplying by thirty the amount of gentian violet broth into which the inoculations were made, that is by using for example 150 cc., the growth of thirty organisms should be prevented. As a matter of fact thirty organisms will grow, with a fair degree of constancy, not only in this amount of gentian violet broth, but in very much larger amounts. When inoculations of thirty organisms were made, for example, into a liter of gentian violet broth, growth occurred in 60 per cent of the flasks, though if the explanation of the phenomena described were purely quantitative it should be necessary to seed 6,000 organisms into this amount of gentian violet broth in order to obtain growth; that is, thirty organisms accomplish not thirty times what one organism accomplishes, but very much more than this. This discrepancy between the work actually accomplished by thirty organisms, and the work which they might be expected to accomplish on a purely quantitative basis is represented by the line *A-B* in Text-fig. 2.

It might be objected that bacteria ought not to be thought of as units exactly equal to one another in efficiency. A given group of thirty might be able to do only fifteen times the work of one very vigorous organism, while another group of thirty might be able to do 60 times the work of a relatively weak organism. If, however, there was any very marked individual variation in the ability of individual organisms to cope with gentian violet, this would have shown itself in occasional growths of vigorous cells when single cell transplants were made. Furthermore, such a variation would hardly explain why thirty cells can grow in a liter of gentian violet broth, and thus do what not less than 6,000 might be expected to do.



TEXT-FIG. 2. The solid line at the bottom of the chart represents the curve of growth of thirty organisms. These grow even in a liter of gentian violet broth. The dotted line represents the curve that would be expected if the growth of groups of organisms depended entirely on the quantity of available gentian violet; in a liter of gentian violet broth 6,000 organisms would have to be seeded to produce growth. The line A-B represents the discrepancy between what actually occurs and what would be expected to occur from a quantitative theory of the difference between the behavior of one cell and that of a group of cells.

That the amount of available gentian violet does, within very narrow limits, play some part in the prevention of growth of single cells is shown in Text-fig. 3. The dotted line A-B represents the results of single cell inoculations into gentian violet broth. It will be seen that single cells never grow in more than 0.8 cc., but that in amounts smaller than this a fair number of positives occur. When 0.1 cc. was used 60 per cent positives were obtained. The corresponding amount of gentian violet broth for thirty organisms would



TEXT-FIG. 3. A-B is the curve of growth for single cells—0 per cent when 5 cc. of broth were used, 60 per cent when 0.1 cc. was used. C-D is the curve of growth for thirty cells, 100 per cent when 5 cc. of broth were used, 60 per cent when 1 liter was used.

be thirty times 0.1 cc., or 3 cc.; yet as a matter of fact 60 per cent positives were obtained with thirty organisms in over 300 times this amount of gentian violet broth; that is, in a liter (Text-fig. 3, line C-D). It is important to observe that the amount of broth used for the experiments, independent of the amount of gentian violet, did not play a part in the results, for in experiments made to determine this point, 75 per cent positives were obtained when single cells were inoculated into a liter of plain broth.

It seems clear, therefore, that thirty cells, instead of being able to accomplish thirty times what one cell can accomplish, are able to accomplish very much more than this. To this discrepancy between the work that thirty cells can do and the work they might be expected to do, on a purely quantitative basis, the term communal activity has been applied. For the present its nature cannot be more accurately defined.

#### SUMMARY.

1. The behavior of a single bacterial cell toward gentian violet differs fundamentally from that of a small group of cells (thirty).

2. The explanation of this phenomenon is not purely quantitative; thirty cells accomplish much more than thirty times what one cell can accomplish.

#### EXPLANATION OF PLATES.

##### PLATE 78.

Fig. 1. Single unstained cells planted on gentian violet agar; no growth.

Fig. 2. Groups of unstained cells of a gentian-negative strain of *B. coli* planted on gentian violet agar. The smaller groups did not grow. The number of cells in the group planted is indicated on each tube.

##### PLATE 79.

FIG. 3. Transplantation of single cells on plain agar for control. In Divisions 1 and 2 two separate plants (*a* and *b*, *c*, and *d*) were made, in the other divisions only one. Six growths were obtained out of seven plants.

FIG. 4. Transplantation of single cells of *B. coli* on gentian violet agar. One cell was planted in each of the ten divisions. No growth occurred.

FIG. 5. Transplantation of single cells of *B. coli* on gentian violet agar. A single cell was planted in each division. *A*, barely visible, *B*, invisible at the end of 24 hours. These two colonies are the only two growths which have ever occurred from single cells in gentian violet media.

##### PLATE 80.

FIG. 6. Single cell transplantations into broth. Strain Z, a gentian-negative strain from a single cell, was used for the seeding. Growth is represented by shading. 80 per cent positives were obtained in the control series (upper row), no positives in the gentian violet broth series (lower row).

FIG. 7. Group transplantations of a gentian-negative strain of *B. coli* (Strain Z) into gentian violet broth. The number of cells seeded is indicated on each tube. Groups below twenty-seven did not grow. Compare the growth of the larger groups with the failure of single cells to grow (see Fig. 6).



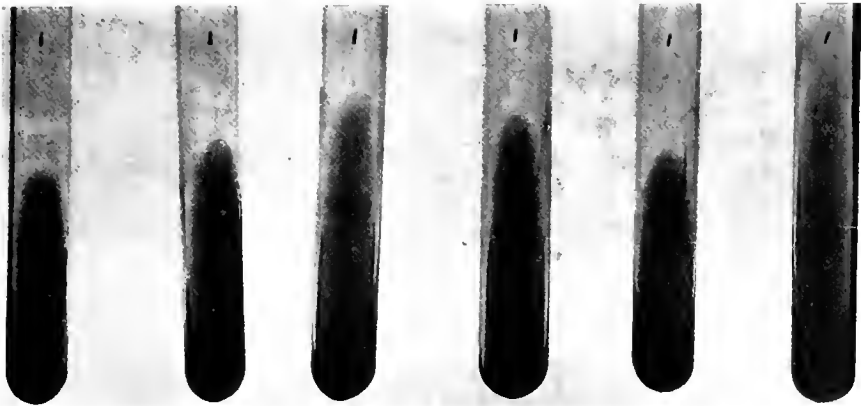


FIG. 1.

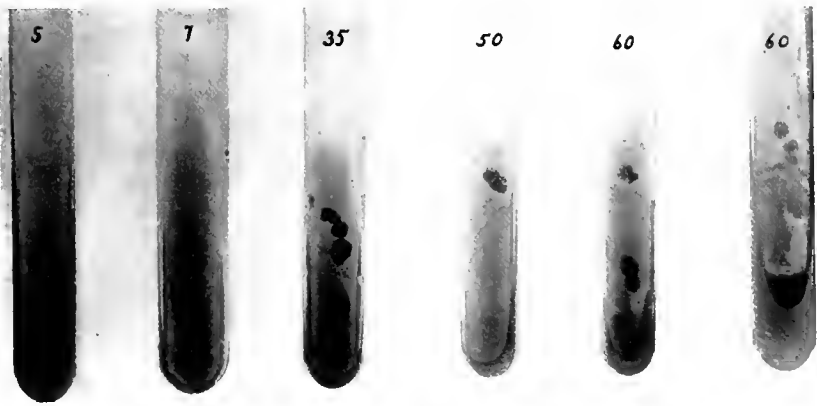


FIG. 2.

(Churchman and Kahn: Communal activity of bacteria.)





592



FIG. 3.



FIG. 4.

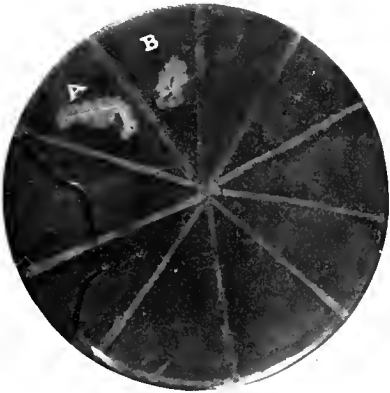


FIG. 5.

(Churchman and Kahn: Communal activity of bacteria.)



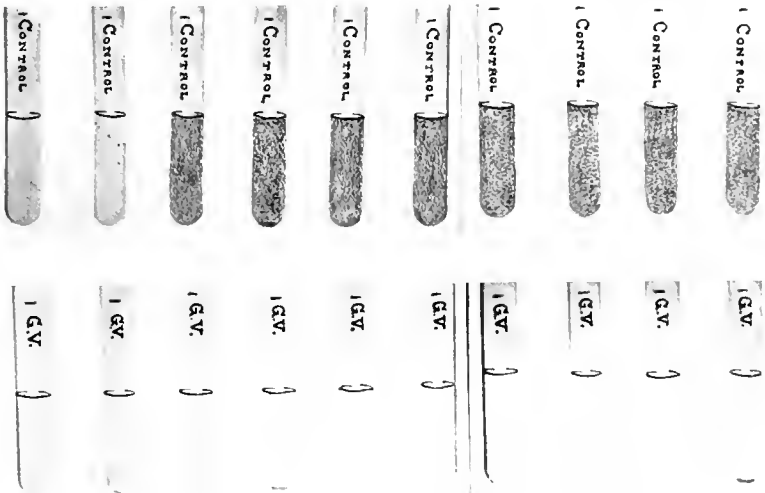


FIG. 6.

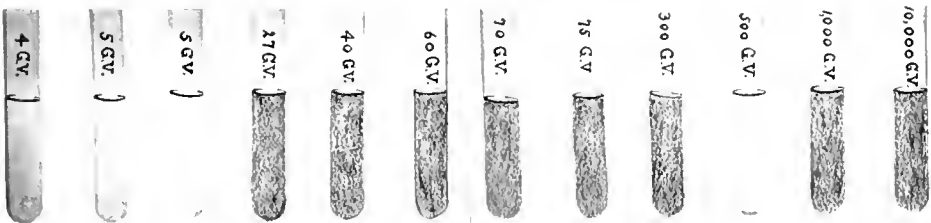


FIG. 7.



## FATE OF THE LYMPHOCYTE.

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(Received for publication, February 21, 1921.)

The normal function of the lymphocyte, as a cell, and the fate of the lymphocytes that enter the blood circulation are questions which apparently have not been solved. It seems to be generally accepted that lymphocytes are produced in the lymphoid tissue of the body and only to a minor extent, if at all, in the bone marrow. From the lymphoid tissue they gain entrance to the efferent lymphatics, and are, in the words of Davis and Carlson,<sup>1</sup> "as much a part of the lymph as the erythrocytes and leucocytes are of the blood." Eventually the lymphocytes, which have entered the lymph stream, in part at least, reach the blood circulation through the major lymph trunks, and chiefly through the thoracic duct. The content of the thoracic duct lymph is high in lymphocytes. Thus, in eleven dogs studied by Rous<sup>2</sup> the leucocytes per c. mm. varied from 2,400 to 13,120. Of these cells, the majority was lymphocytes. Davis and Carlson<sup>1</sup> found the number in dogs to vary from 1,000 to 30,000 leucocytes per c. mm., of which from 95 to 100 per cent were lymphocytes.

While it is not certain that the rate of flow of lymph from the thoracic duct through a cannula represents the normal flow into the jugular vein, especially since the animal is anesthetized,<sup>3</sup> yet, when such flow remains fairly constant over a period of hours, one must assume that it approximates the normal flow. If, with this point in mind, we calculate, from fourteen dogs in Rous<sup>2</sup> series on a basis of the lymphocyte content of the thoracic duct per cubic millimeter and the rate of flow during fixed intervals of his experiments, we find a surprisingly large number, 3,300,000,000 cells, as the total number that

<sup>1</sup> Davis, B. F., and Carlson, A. J., *Am. J. Physiol.*, 1909-10, xxv, 173.

<sup>2</sup> Rous, P., *J. Exp. Med.*, 1908, x, 238, 329, 537.

<sup>3</sup> All operations were performed under ether anesthesia.

would enter the blood stream during 24 hours. This is a larger number than that of lymphocytes present in the blood stream at any one time. Davis and Carlson agree with this estimate in their statement that "many more lymphocytes enter the blood with the lymph in the course of 24 hours than can be found in the blood at any given time."

Given these findings, then, of the entrance of an enormous number of lymphocytes into the blood each day, and the further findings of a relatively constant count of such cells in the circulating blood, a problem of great interest is presented as to the fate of these cells. As many cells must disappear from the circulation each day as are introduced into it, or we would find not a constant count but an ever increasing accumulation of these cells in the blood stream. It is with this problem that the present paper is concerned. There would seem at the outset only three possibilities as to the fate of the lymphocyte: (1) It might be changed in development into some other type of leucocyte. (2) It might disintegrate in the blood stream. (3) It might leave the blood stream. Since the first possibility seems remote, as a result of modern work on hematology, we think that it may be dismissed from consideration and have therefore confined our work to the two other possibilities.

#### EXPERIMENTAL.

Our work has been done on rabbits, and although chiefly the fate of the lymphocyte has been studied, a few observations have been made on these cells in the thoracic duct and on the rate of flow. Direct counts, obtained not on freely flowing fluid, but from fluid from the duct immediately after the death of the animal, showed from 20,000 to 50,000 cells per c. mm. in four animals, with a proportion of 80 per cent of small lymphocytes to 20 per cent of larger lymphoid cells in counts of 1,000 cells. Granular polymorphonuclear cells and red cells do not appear as normal constituents of the thoracic duct lymph of the rabbit. Agglutinated masses of poorly staining lymphocytes, apparently disintegrating, are quite numerous in stained films of duct lymph. The possible significance of these will be referred to later. We have, in one of two attempts, succeeded in catheterizing the thoracic duct in the rabbit and we obtained lymph flow at the rate of 1 cc. in 10 minutes, in an etherized animal. This does not

necessarily represent the normal rate of flow. The animal is in an abnormal position; the rate and depth of its respiration are abnormal and seem to determine the progress of the column of fluid through the cannula. As the cannula must be of almost capillary size, capillary attraction must be a factor in rate of delivery; on the other hand, the viscosity of the lymph prevents free dropping from the end of the cannula. Withal, it may be assumed that a very considerable number (a billion or more) of lymphocytes enter the blood from the thoracic duct in the course of a day.

*Determination of the Rapidity of the Disappearance of Lymphocytes from the Blood.*

In a series of experiments on the rabbit, we attempted to cut off the inflow of lymphocytes into the circulation and thus to determine the rapidity of their disappearance from the blood. As a primary procedure, animals were splenectomized under ether anesthesia to exclude the direct entrance into the blood of lymphocytes from the Malpighian corpuscles. Then, after a sufficient interval for recovery and for return of the leucocyte formula to approximately normal, the thoracic duct was occluded by a ligature which included both the thoracic duct and the jugular vein just below the entrance of the former into the jugular. In later experiments the neck lymphatic trunks on the right and left sides were also tied. Since the results of these experiments were uniform, as far as the trend of the change in the lymphocyte count is concerned, although there was a variation in degree, it does not seem necessary to report the series in detail, especially as the procedure is not new and the results do not differ from those of others. The two protocols below illustrate the results.

*Rabbit 1.*—Male; weight 1,500 gm. December 1, 1920. Spleen removed under ether and with aseptic precautions. Recovery, apparently without complications. December 9. With careful surgical technique one ligature was placed around thoracic duct and left jugular, and a second about the main right cervical lymph trunk. Blood counts, made from the right ear with free flow of blood, are given in Table I.

In this animal there is a drop in lymphocytes of 1,826 per c. mm. in 5 hours and 1,955 in 10 hours after partially cutting off the supply,

with a gradual return toward the normal number. On the basis of 75 cc. (5 per cent of body weight) of blood this primary drop represents the disappearance from the blood stream of 136,950,000 lymphocytes in 5 hours. The simultaneous drop in polymorphonuclears is definitely due to migration into the operative field.

TABLE I.

Time.	Total No. of leucocytes.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
		Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
Before splenectomy.....	8,000	28	2,240	61	4,880	6	480	5	400
“ tying thoracic duct.	8,500	34	2,890	60.5	5,142	2.5	212	3	255
5 hrs. later.....	5,600	19	1,064	70	3,920	3	168	8	448
10 “ “ .....	5,500	17	935	66	3,630	4	220	13	715
24 “ “ .....	8,000	24	1,920	58	4,640	5	400	13	1,140
2 days “ .....	7,600	23	1,748	59	4,484	4	304	14	1,064
3 “ “ .....	6,900	22	1,516	61	4,209	4	276	13	897
4 “ “ .....	8,300	22	1,826	60	4,980	5	415	13	1,079
6 “ “ .....	8,100	25	2,116	58	4,698	4	324	12	972

TABLE II.

Time.	Total No. of leucocytes.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
		Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
Before splenectomy.....	4,100	51	2,091	41	1,678	3	123	5	205
“ tying thoracic duct.	9,000	53	4,770	36	3,240	5	450	6	540
6 hrs. later.....	4,500	8	340	85	3,825	4	180	3	135
12 “ “ .....	6,200	5	310	86	5,332	5	310	4	248
24 “ “ .....	6,700	15	1,005	73	4,891	5	335	7	469
48 “ “ .....	12,000	11	1,320	78	9,160	2	240	9	1,080
4 days “ .....	9,700	13	1,251	72	6,984	3	291	12	1,164
6 “ “ .....	7,600	25	1,900	66	5,016	3	228	6	456
10 “ “ .....	7,700	40.5	3,118	50.5	3,888	3.5	277	5.5	423

*Rabbit 2.*—Male; 2,000 gm. December 7, 1920. Splenectomy. Recovery took place without apparent complications and none was found at subsequent autopsy. January 5, 1921. The thoracic duct and jugular were included in a ligature; a second was placed above to include the left cervical trunk; the right cervical main trunk was tied, and an attempt was made to dissect free the lymphatic trunks in the neighborhood of the right jugular and subclavian junction. The tissues were closed in layers by interrupted sutures. The blood counts in this animal are given in Table II.



In this animal, with probably better exclusion of lymphocytes, there is a drop in the number of lymphocytes of 4,430 per c. mm. in 6 hours, or, on a basis of 100 cc. of blood, a total disappearance from the circulation of 443,000,000 lymphocytes, a fact well confirmed by the count after 12 hours. This is followed, as in the previous animal, by a gradual return toward normal.

In this last animal the most successful exclusion of lymphocytes was obtained. Success depends upon the character of the main lymph trunks. The rabbit is not satisfactory for such experiments since there is frequently a right thoracic duct as well as a left, as pointed out by Gage<sup>4</sup> and as our injections of experimental animals, post mortem, have indicated. There are also many anastomosing lymph vessels near the entrance of the duct into the jugular. Injection of the thoracic duct through the capsule of the mesenteric lymph glands in one of our animals showed anastomosing vessels leading through the thymus toward the right side. It is through the utilization of such channels that the lymphocyte count in the blood is gradually restored.

#### *Fate of the Lymphocyte.*

With these indications of a large supply of lymphocytes and, as is consistent with this, a rapid disappearance of these cells when the supply is cut off, the question as to their fate is still left unanswered. Do they disintegrate in the blood stream or do they leave it? Occasional disintegrating lymphocytes are found in stained blood films made from normal and from diseased individuals, and it seems unquestionable that some lymphocytes may go to pieces in the blood. In an attempt to answer the question as to whether this is the fate of the large majority of lymphocytes, an experiment was performed, which, though not leaving them in a strictly physiological state, simulated this condition as closely as possible. After the number of lymphocytes in the freely flowing blood from the marginal vein of the right ear had been established, a section of the right jugular vein full of blood was isolated between carefully applied double ligatures. Counts of the cells in this section were made 6 hours later in some

<sup>4</sup> Gage, S. H., in Buck, A. H., Reference hand-book of the medical sciences, New York, 2nd edition, 1902, v, 624.

animals and 24 hours later in others. These experiments are open to objection since the cells are probably exposed to a reduced oxygen tension in the stagnant blood and the varied ameboid motion of cells and the settling of corpuscles cause an uneven distribution at the end of 24 hours which makes the counts somewhat questionable. Yet, as they have a bearing on the length of life of the lymphocyte, three counts are given (Table III).

TABLE III.

Rabbit No.	Site of count.	Total count.	Lymphocytes.		Neutrophils.		Basophils.		Large mononuclears.	
			Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.
3	Count from right ear.	7,800	41	3,198	52	4,056	5	390	2	156
	“ “ jugular section 6 hrs. after ligation.	7,250	63	4,567	30	2,175	5.3	384	16	116
4	Count from right ear.	7,250	52	3,770	37	2,682	8	580	4	295
	“ “ jugular section 24 hrs. after ligation.	4,600	62	2,852	32	1,372	4	184	2	92
5	Count from right ear.	9,000	36	3,240	50	4,500	2	180	13	170
	“ “ jugular section 24 hrs. after ligation.	6,800	53	3,604	40	2,720	2	136	5	340

In spite of the inaccuracies of the method, there is apparently a greater disappearance of polymorphonuclear cells than of lymphocytes. This was explained by histological sections made through the vein wall, which showed not that these cells were destroyed, but that there was a marked migration of them toward the operative field. In the stained film from the jugular blood, the most striking feature is the fact that the lymphocytes show no tendency toward disintegration but are as sharply stained and clear-cut in the differentiation of nucleus and protoplasmic rim as in films from the freely circulating blood. We believe that the experiments, though faulty, indicate that there is not the rapid disintegration of the cells which would be necessary to explain the rate of their disappearance from the blood when the supply is cut off. They must leave the blood stream.

Since the ameboid motion of lymphocytes is generally accepted, there is no objection *a priori* to the proposition that they leave the blood stream under normal conditions in the numbers demanded by the figures given. The question arises as to where they go. The small accumulations of lymphocytes found in the organs generally will not account for the vast number that leaves the blood vessels, unless the cells are destroyed in the tissues as rapidly as we postulated their destruction in the blood stream, and of this there is no evidence in normal organs. Without destruction, of necessity there would be an increasing accumulation in these organs such as may be seen in lymphocytic leucemias.

A search of the organs in histological section leads to the conclusion that the great majority of these lymphocytes enter the mucosa of the gastrointestinal tract and, in addition, pass through it into the intestinal lumen. In sections of rabbit intestine removed immediately after the death of the animal and fixed rapidly in formaldehyde without opening or disturbing the contents, the number of cells in the mucosa, between the cells of the intact epithelial lining, and in the intestinal content is surprising, not only in the parts of the tract where lymphocyte production takes place, as in the lower ileum and the appendix, but also high in the duodenum. If the 40 or 50 lymphocytes which may be counted between the epithelial cells of the tip of a duodenal villus in a 10 micron section are multiplied by its probable relation to the surface area of the intestine, there is no difficulty in accounting for all the lymphocytes that disappear from the circulation. Where there is local production, as in the lymphoid nodules of the appendix, several hundred lymphocytes and large mononuclears may be found in a section through the mucus of a single crypt. In a section of the intestine made without disturbing the contents of the lumen, collections of lymphocytes and larger mononuclear cells are numerous close to the mucosa, especially in the small intestine, even, as stated above, high in the duodenum. In the rabbit colon, which contains inspissated fecal masses, there is little or almost no migration of lymphocytes. The scarcity of polymorphonuclear leucocytes throughout the intestine is as striking as the presence of the mononuclear cells.

Several attempts were made to count the free lymphocytes in the intestinal content by taking up into a calibrated capillary tube a

known amount of fluid close to the mucosa, spreading it over a small part of a cover-glass, staining, and counting the total number of cells. Such a count gave 100 definite lymphocytes per c. mm. for the upper duodenum, 480 for the ileum, and 700 for the appendix. These figures are without significance when taken in relation to the total volume of the intestinal contents, as the lymphocytes are not evenly distributed throughout the contents, at least in recognizable form, but lie close to the mucosa. However, they indicate that large numbers of lymphocytes enter the lumen normally. In fact, one may speak of a normal excretion of lymphocytes from the intestinal mucosa. This takes place not only from the blood stream but from the lymphoid nodules and patches, which are shown by sections to be delivering lymphocytes to the intestine on one side, and to the efferent lymphatics on the other. The latter cells must take an indirect route through the thoracic duct and the blood stream to reach the lumen. To a slight degree the same process is visible in the bronchial mucosa of the rabbit.

It would seem then that under normal conditions the lymphocyte fulfills its function to a large degree upon the surface of the intestinal mucosa. To attempt to designate that function would, with our present knowledge, be a matter of almost pure speculation. In attacking the problem, it is difficult not to be influenced by the immunity of the intestinal mucosa to the countless bacteria within its lumen and to their toxins. The lymphocyte is not phagocytic, but there are many indications that it may affix toxins, and among these may be cited the agglutination of lymphocytes noted in the thoracic duct.

#### CONCLUSION.

Although the count of circulating lymphocytes in the blood stream remains constant, more lymphocytes enter the blood from the thoracic duct during 24 hours than are present in the blood at any one time. This excess of lymphocytes is not destroyed in the blood stream. The cells migrate from the blood vessels into the mucous membranes and through them to their surface. This occurs chiefly in the gastrointestinal tract, and it is apparently in the mucosa and especially within the intestinal lumen that the function of the lymphocyte is normally performed.

## RELATION OF ANTIBODY AND ANTIGEN TO SERUM DISEASE SUSCEPTIBILITY.

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(Received for publication, January 20, 1921.)

Longcope and Rackemann<sup>1</sup> have reported observations on the precipitin, anaphylactic antibody, and cutaneous hypersensitiveness in individuals to whom serum had been administered therapeutically. In the course of these studies, two patients were encountered who developed no serum disease. It was found that in the blood of these patients, no precipitin was demonstrable. The studies reported in the present paper were undertaken with a three-fold purpose: (1) to pursue further the investigation of the relations between precipitin formation and the symptoms of serum reactions; (2) to determine whether or not the disappearance of horse serum from the circulation can be brought into relation with precipitin formation or the symptoms; and (3) to investigate further the factors concerned in the non-susceptibility of certain individuals to serum sickness.

### *Method.*

The presence in the patients' serum of the antipneumococcus or antimeningococcus horse serum, which we refer to as precipitinogen, was determined by specific precipitation with the serum of rabbits immunized against horse serum. This anti-horse rabbit serum was used in all the tests without inactivation, and without preservatives, but was diluted with an equal volume of normal salt solution. On each day on which tests were done, the rabbit serum was titrated

\* The participation of Dr. Leake in the investigation reported in this paper was made possible by the donation of a fund by Mrs. Arthur W. Swan.

<sup>1</sup> Longcope, W. T., and Rackemann, F. M., *J. Exp. Med.*, 1918, xxvii, 341.

against dilutions of normal horse serum, and to insure the specificity of the reactions, it was titrated against several dilutions of normal human serum. Owing to the frequent occurrence of prozone phenomena, it was found necessary to set up several dilutions of each of these controls, because sometimes an anti-horse rabbit serum which gives no precipitation with human serum in dilutions of 1:2 or 1:10 will give a definite clouding or actual precipitate in dilutions of 1:100 or 1:1,000. Obviously, any such undetected non-specific reactions would lead to false conclusions as to the presence of precipitinogen in the patients' serum.

In the determination of precipitinogen, there is another possible source of error which must be guarded against. It is, of course, well known that an immune serum may contain both precipitin and precipitinogen without forming a precipitate.<sup>2,3</sup> But if two such sera are mixed, precipitation follows. In determining the titer, therefore, of the precipitinogen in the patient's serum, it is important to use only immune sera which have been tested and found to be free from precipitinogen. Otherwise when precipitin is present in the serum of the patient, it will precipitate these persisting traces of precipitinogen in the immune rabbit serum and make it appear that precipitinogen is still present in the circulation of the patient. The absence of precipitinogen from the immune rabbit serum is demonstrated by securing two anti-horse rabbit sera which, when titrated against each other, produce no precipitation.

Determinations of the precipitin in the patient's serum were made by the usual method of setting up the patient's serum, diluted with an equal volume of salt solution, against several dilutions of horse serum. In all the tests, both for precipitin and precipitinogen, a constant amount of the precipitating serum was added to increasing dilutions of precipitinogen.

Usually the tests were done on the same day that the blood was drawn, but in a few instances the serum was kept in the ice box over night before being used. After being set up, the tubes were placed in the water bath for 1 hour at 37°C. and then in the ice box over night, when the final reading was made.

<sup>2</sup> Weil, R., *Proc. Soc. Exp. Biol. and Med.*, 1914-15, xii, 37.

<sup>3</sup> Denzer, B. S., *J. Infect. Dis.*, 1916, xviii, 631.

The delicacy of these tests is considerably increased if the serum is perfectly clear. For several hours after an ordinary meal, the serum is so clouded with fat that accurate readings are impossible. Whenever possible, therefore, the blood was drawn after a 14 hour fast. This almost always insures a perfectly clear serum.

### *Material.*

By the methods described above, we have studied twenty-one patients to whom serum was administered for therapeutic purposes either intravenously or intraspinally in amounts varying from 34 to 630 cc. There were, however, two patients in this group who, we felt, should be excluded from analysis because of complicating factors, affecting to an unknown degree the phenomena under consideration. One of these was a patient with lobar pneumonia, Type I, syphilis, and a severe amebic colitis. As he was having frequent watery stools, it seemed probable that he might be eliminating the foreign serum through the intestinal mucosa. If such were the case, the precipitinogen would disappear from the circulation earlier than it would if only the usual mechanism for elimination were operating. The same consideration holds for the other patient excluded from analysis. This patient was a child 5 years old with Pneumococcus Type I peritonitis and empyema. Laparotomy and thoracotomy were done and the abdomen and pleural cavity were drained. It was therefore thought to be highly probable that she was eliminating precipitinogen through the abdominal and thoracic sinuses and that conditions were not comparable to those existing in the other patients. It is noteworthy that the discrepancy observed in these two patients in the interrelations of precipitin, precipitinogen, and symptoms was merely the unusually early disappearance of precipitinogen from the circulation.

The nineteen patients remaining for consideration varied in age from 13 to 56. There were thirteen males and six females. They were treated with antipneumococcus or antimeningococcus serum in amounts varying from 34 to 630 cc. Eighteen were patients with lobar pneumonia, Type I, and one was a patient with meningococcus meningitis. Only one of the patients showed cutaneous hypersen-

sitiveness prior to the therapeutic administration of serum. The single individual who was hypersensitive when he came under observation will be referred to again. These data regarding the patients studied are shown in Table I.

TABLE I.

Patient No.	Age.	Sex.	Diagnosis.	Amount of serum.	Method of administration.	Intracutaneous test with horse serum before administration of serum.
	yrs.			cc.		
1	56	M.	Lobar pneumonia.	100	Intravenously.	Negative.
2	20	"	" "	200	"	"
3	39	F.	Meningococcus meningitis.	95	Intraspinaly.	"
4	25	M.	Lobar pneumonia.	500	Intravenously.	"
5	25	F.	" "	300	"	"
6	24	"	" "	320	"	"
7	32	M.	" "	480	"	"
8	20	"	" "	300	"	"
9	37	"	" "	630	"	"
10	25	"	" "	370	"	"
11	32	F.	" "	270	"	"
12	22	M.	" "	34	Subcutaneously, 2 cc. Intravenously, 32 "	Positive.
13	23	F.	" "	500	"	Negative.
14	13	M.	" "	180	"	"
15	25	"	" "	380	"	"
16	19	"	" "	385	"	"
17	28	"	" "	520	"	"
18	26	"	" "	200	"	"
19	32	F.	" "	500	"	"

## RESULTS.

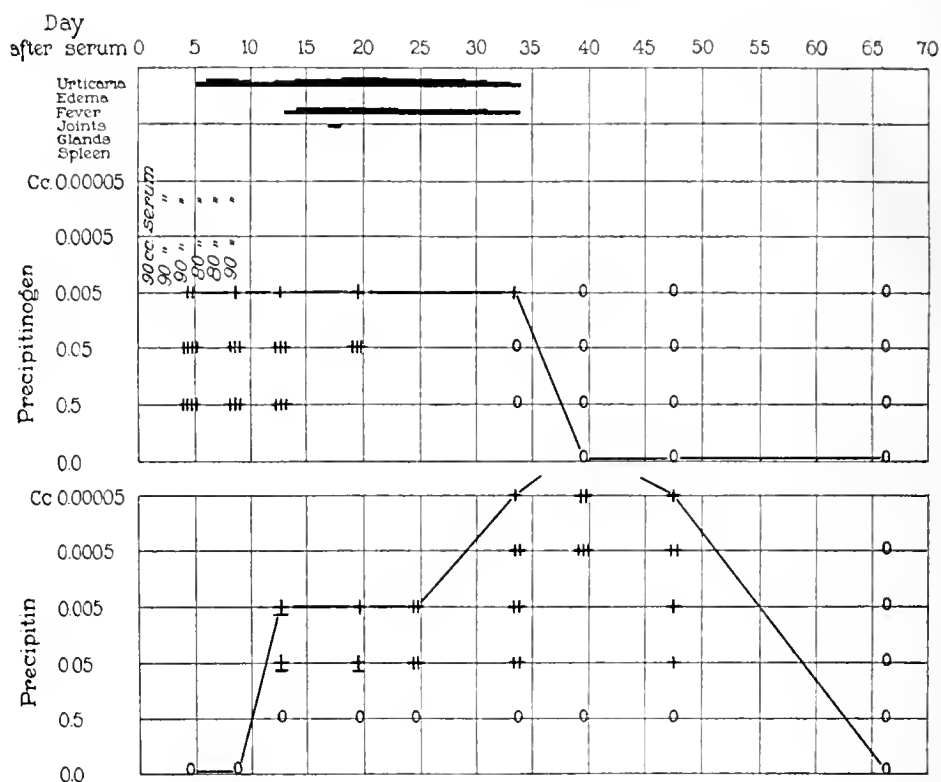
Analysis of the results obtained in this series of nineteen patients shows that from the point of view of circulating precipitin, persistence of precipitinogen, and severity of symptoms, they may be divided into three groups.

*Group 1.*—Eleven of the nineteen patients fall quite sharply into one group. This group has the following characteristics: (1) Severe serum disease. If the onset of the serum reaction is considered to be marked by the appearance of an eruption, edema, or arthralgia, and



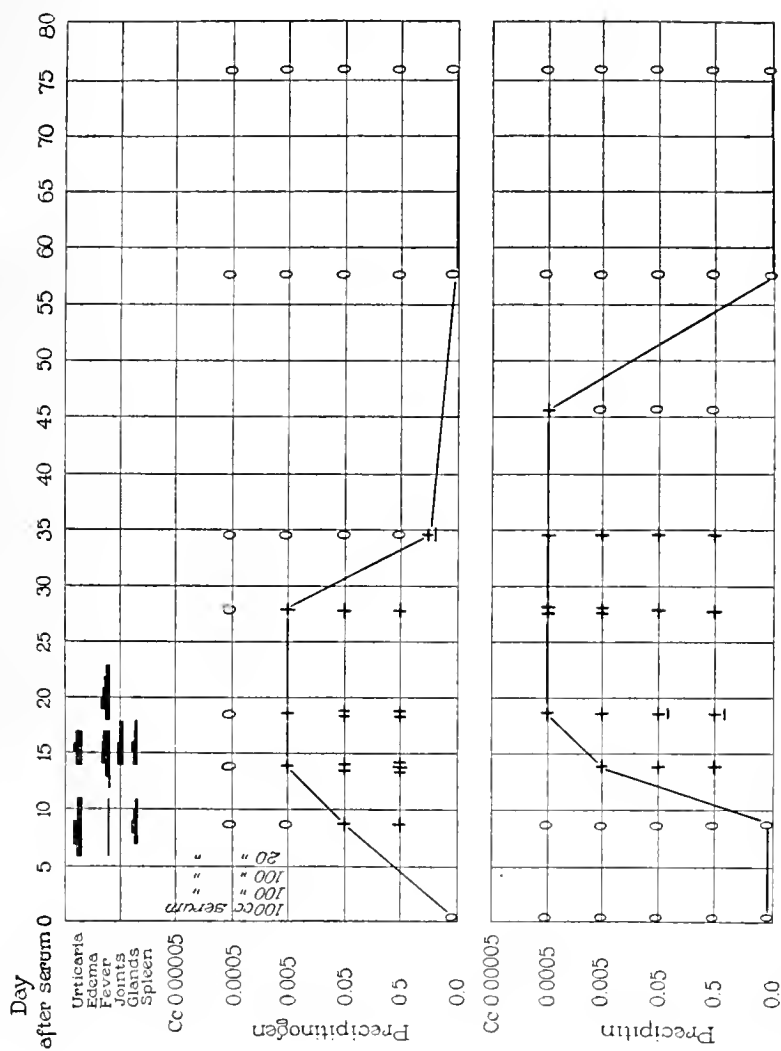
the termination of the serum reaction to be marked by the disappearance of these symptoms, ten of the eleven patients in this group had serum disease lasting 7 days or longer. The other patient had an urticarial eruption lasting 6 days. (2) A relatively high titer of precipitin appearing first or rising to the crest of the curve near the time that symptoms are subsiding. In only one of the patients in this group was the precipitin titer at the height of the curve less than 1:2,000. (3) Disappearance of precipitinogen from the circulation near the termination of the symptoms of serum disease. The usual form of curve for precipitinogen was a steady persistence at a high level until the precipitin curve rose sharply to its high point; a steep decline in the precipitinogen curve then occurred. Efforts were repeatedly made to demonstrate the presence of the foreign serum in the urine, but these tests were, without exception, negative. It seems probable, therefore, that the circulating precipitinogen is not eliminated by the kidneys, at least not in a form demonstrable by specific precipitation.

The characteristic interrelations of the three factors under consideration—precipitin, precipitinogen, and symptoms—are shown in Text-figs. 1 to 4. In Text-fig. 1, it is seen that the serum reaction began on the 5th day and persisted until the 34th day, that precipitin first appeared in the circulation on the 12th day, and rose to the crest of the curve synchronous with the disappearance of horse serum from the circulation and near the time that the symptoms subsided. Text-figs. 2 and 3 show very similar relations between precipitin, precipitinogen, and symptoms. Text-fig. 4 also conforms to this type but is of special interest because this patient had twice previously been treated with horse serum; 7 years before, he had been given diphtheria antitoxin without any reaction, and 2 years previously, he had meningococcus meningitis and was given antimeningococcus serum intraspinally. About 2 weeks later, he developed a severe attack of urticaria and his eyes were swollen. When we first saw him, he gave a positive intracutaneous reaction to horse serum and an attempt to desensitize him by the Besredka method of fractional doses was attempted. After a total of 34 cc. had been given, he developed an immediate reaction consisting of erythema and urticaria. This persisted for 24 hours, then 2 days later, that is on



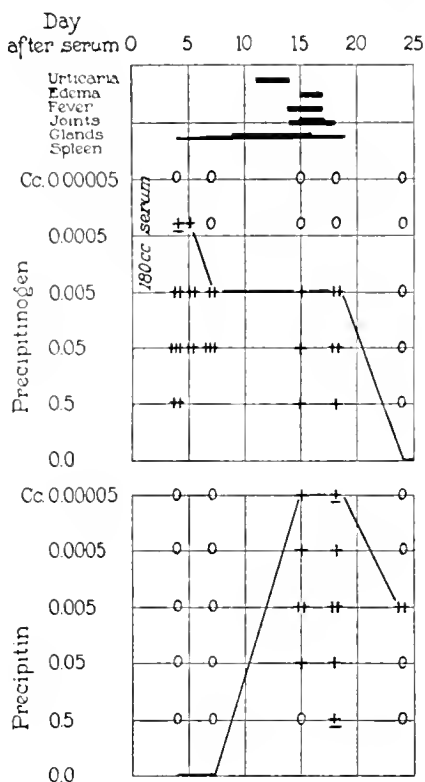
TEXT-FIG. 1. Patient 17 (Table I). Lobar pneumonia, Type I. Serum injections were made on the first 4 days.

In all the text-figures the abscissæ represent the time in days following the first administration of serum. The ordinates represent the dilutions of antigen used in the tests for precipitin and precipitinogen. At the top of the figure the symptoms of the serum reaction are shown in black. The results of the precipitin reactions in the various dilutions employed are shown by + and 0 signs. In the text-figures in which the amounts of serum for the different injections are shown it has sometimes been necessary, in order to make the lettering large enough to be legible, to include a larger number of days for serum injections than was actually involved. When this has been done the actual number of days on which serum was given, is indicated in the legend of the figure.



TEXT-FIG. 2. Patient 6 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

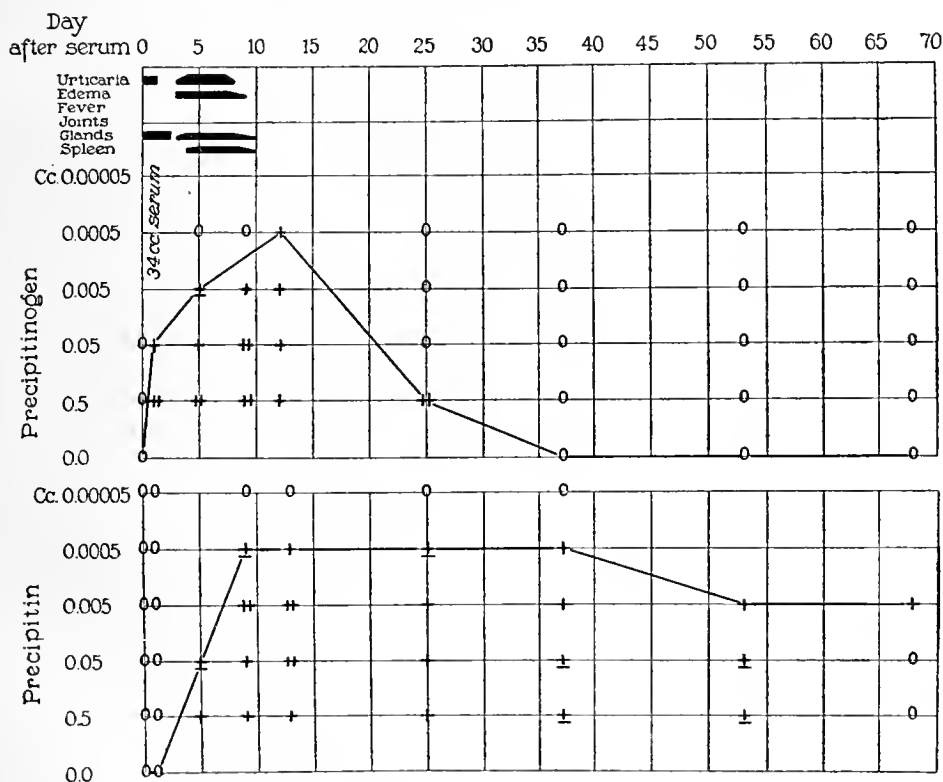
the 3rd day following the serum injections, an accelerated reaction developed, characterized by a morbilliform, urticarial, and purpuric eruption, edema, and enlargement of the lymph nodes and spleen. The particularly interesting feature was the accelerated appearance of precipitin, parallel with the accelerated symptomatic reaction. It is readily seen in Text-fig. 4 that the interrelations between pre-



TEXT-FIG. 3. Patient 14 (Table I). Lobar pneumonia Type I.

cipitin, precipitinogen, and symptoms were essentially the same in this reinjected patient, who showed immediate and accelerated reactions, as in patients receiving serum for the first time. The chief difference appears to be a shortening of the incubation period both for symptoms and for antibody formation. This is comparable to the early appearance of antibodies upon reinjection of rabbits pre-

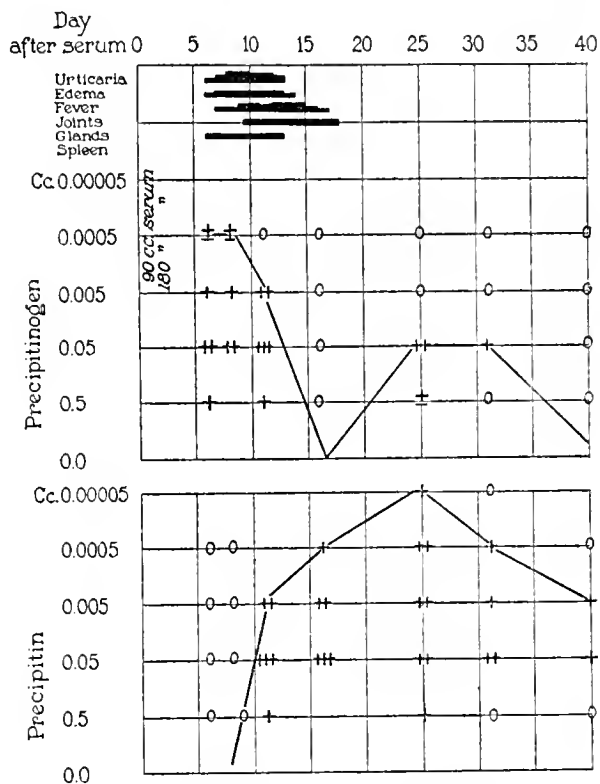
viously sensitized, but without antibody free in the circulation at the time of reinjection. The other seven patients in this group show similar relations between precipitin, precipitinogen, and symptoms. No two are exactly alike, nor would one, from analogy with antibody formation in animals, expect them to be so. The duration of the symptoms, the rapidity with which the precipitinogen disappears,



TEXT-FIG. 4. Patient 12 (Table I). Lobar pneumonia.

and the height of the precipitin curve show considerable variations, but in an unmistakable way, the characteristics of this group are manifest in all of the eleven patients. However, there are two patients in this group presenting a phenomenon demanding further comment. In Text-fig. 5, it is seen that following the rise of precipitin on the 11th day, the precipitinogen completely disappears from

the circulation, but appears again later, and after 9 days gives a positive reaction in dilutions up to 1:200. In following precipitin and precipitinogen in the circulation of rabbits, we have several times encountered a similar phenomenon. It seems as if under certain conditions the union of precipitin and precipitinogen were

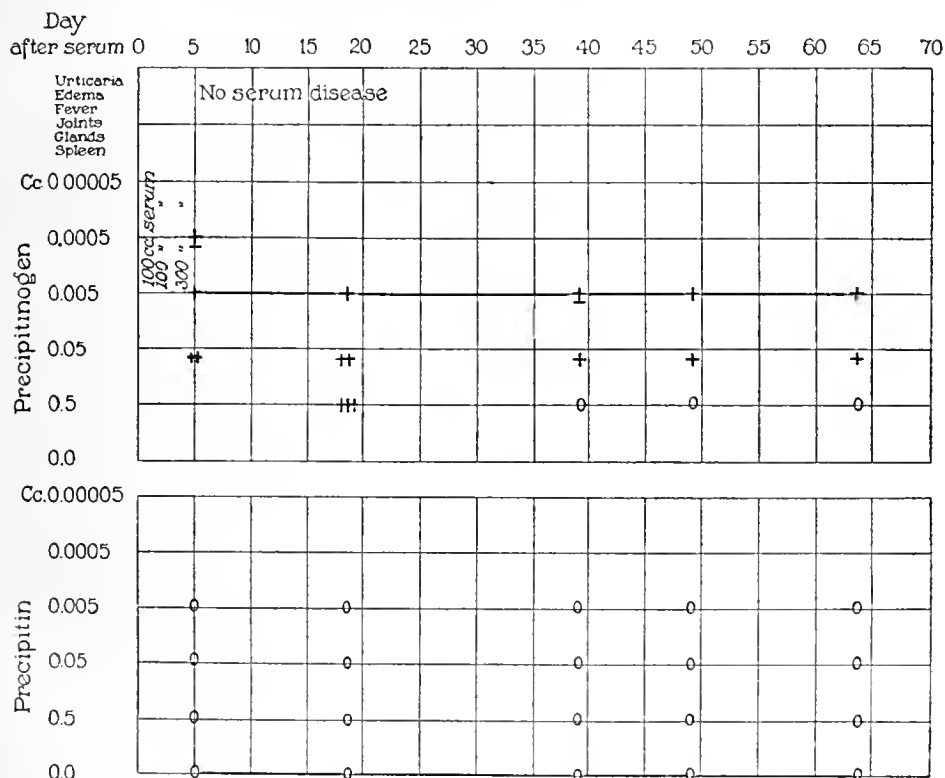


TEXT-FIG. 5. Patient 11 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

unstable and after being bound for a time by precipitin, the precipitinogen is subsequently released and circulates freely in the blood stream. We have no adequate explanation to offer for this reappearance of precipitinogen.

*Group 2.*—The patients in this group, four in number, form an interesting contrast with those just described. Instead of a severe

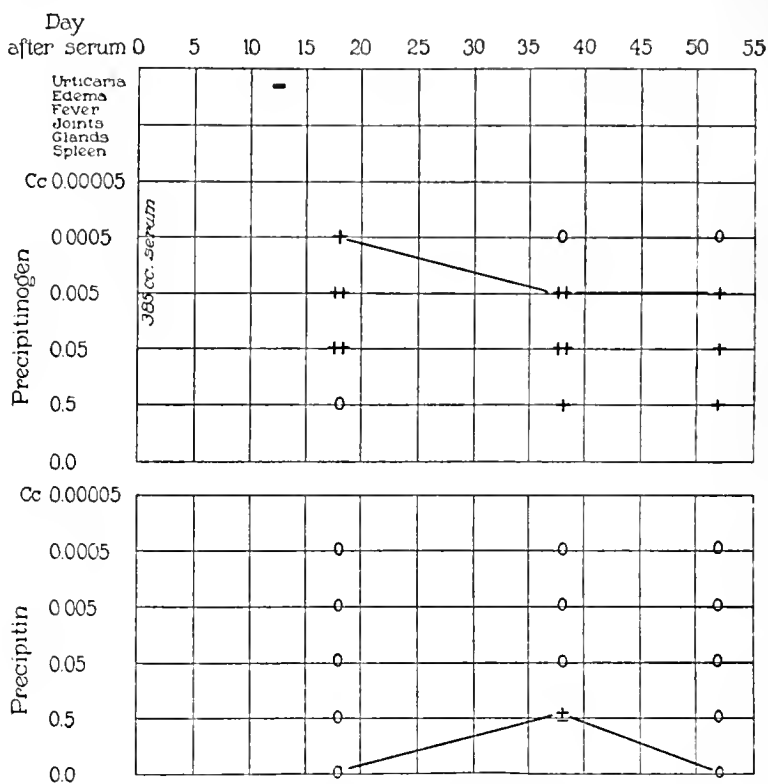
serum reaction, there is none at all or only very mild symptoms; instead of a high titer of precipitin, none is demonstrable in the circulation, or perhaps traces are present for a short period; instead of an early disappearance of precipitinogen, in each of the four patients of this type, there was a positive reaction for precipitinogen as long as the patient could be kept under observation—from 52 to 67 days.



TEXT-FIG. 6. Patient 13 (Table I). Lobar pneumonia Type I. Serum injections were made on the 1st, 2nd, and 4th days.

Text-figs. 6 to 8 illustrate the relations between precipitin, precipitinogen, and symptoms which characterize this group. In Text-fig. 6, it is seen that this patient, although given 500 cc. of serum intravenously, had no symptoms of serum disease, that precipitin did not appear in the circulation, and that horse serum was still present in the circulation 63 days after the first administration of serum. Text-

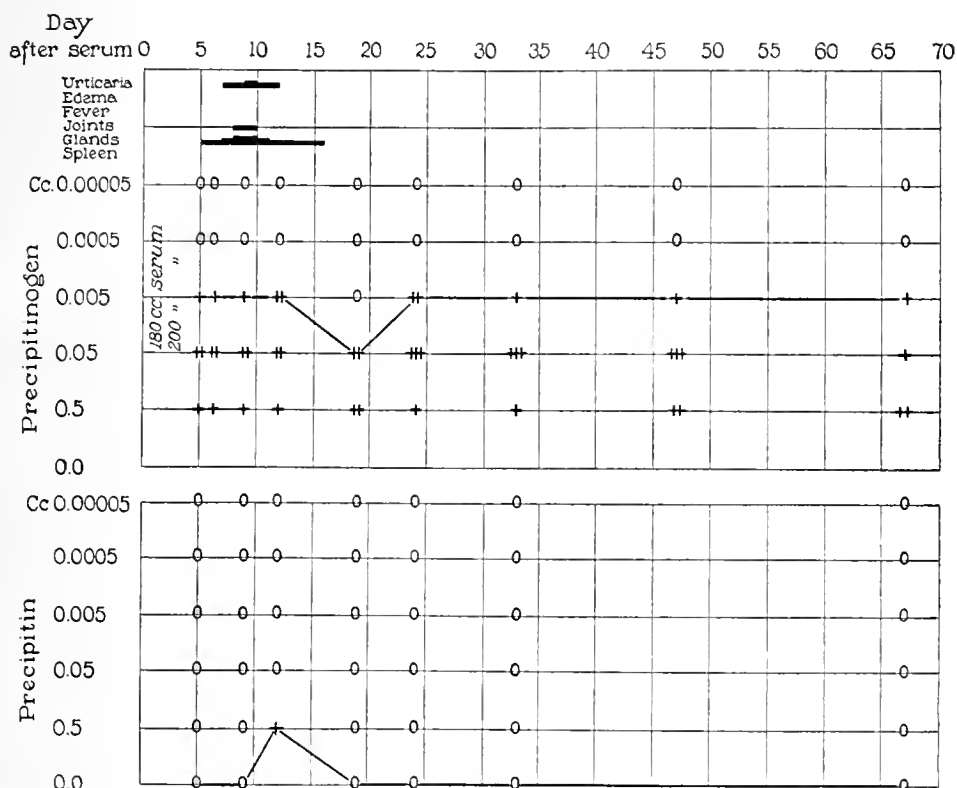
figs. 7 and 8 show relations of the same type between the three factors under consideration, although in each of these patients, there was a mild serum reaction and a transient appearance of precipitin in the lowest dilution.



TEXT-FIG. 7. Patient 16 (Table I). Lobar pneumonia Type I.

*Group 3.*—The four patients remaining do not fall definitely into either of the two groups, the characteristics of which have been described. Three of these four patients, however, had mild serum disease and transient or scanty precipitin in the circulation. One of these three is quite clearly a type intermediate between the two groups already described. Text-fig. 9 shows that this patient had a mild serum reaction, a very low titer of precipitin in the circulation, and a curve for precipitinogen which fell slowly, being intermediate

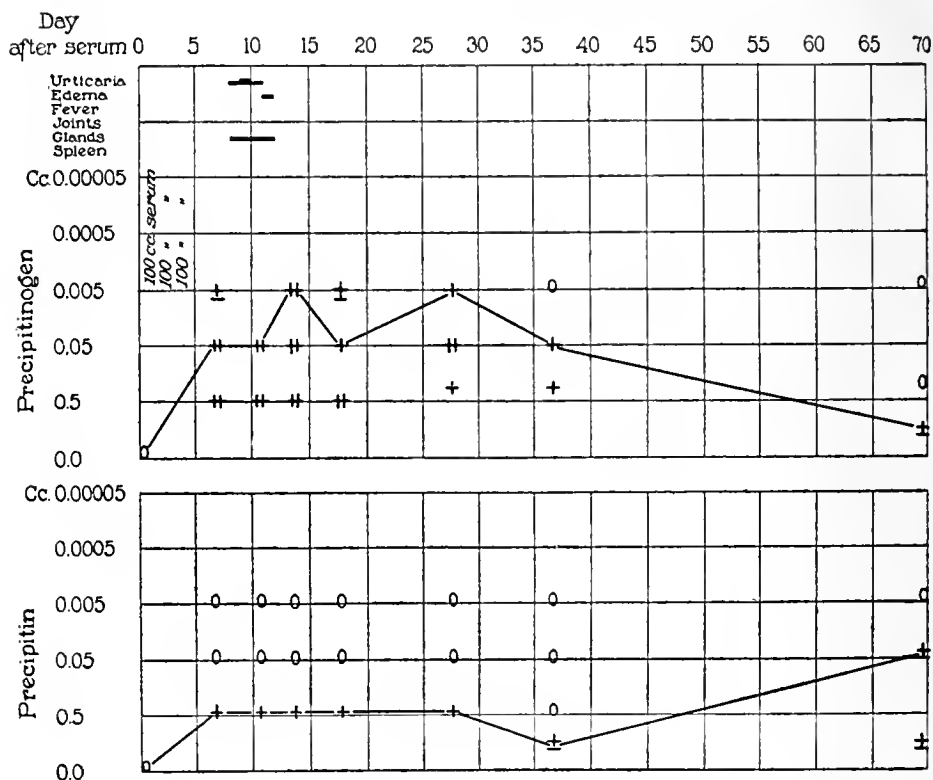




TEXT-FIG. 8. Patient 15 (Table I). Lobar pneumonia Type I. Serum injections were made on the first 2 days.

between the steep decline characteristic of the severe serum disease group and the steady persistence of the precipitinogen characteristic of the group which had no serum disease, or only very slight symptoms. One other patient was perhaps of this same type, but he was lost to observation before the curves could be completed. He had mild serum disease, transient appearance of precipitin, and a precipitinogen curve which showed a decline after the temporary appearance of precipitin, but did not reach the base-line. Owing to the fact that observation of this patient could not be continued, it was not possible to demonstrate that precipitin reappeared in the circulation or to determine when precipitinogen disappeared.

The third patient in this group of four which showed atypical curves was a patient with meningococcus meningitis who was given 95 cc. of antimeningococcus serum intraspinally. She had a very mild serum disease, a low titer of precipitin, and a curve for precipitinogen resembling that found in the patients with severe serum



TEXT-FIG. 9. Patient 5 (Table I). Lobar pneumonia Type I; pregnancy. Serum injections were made on the first 3 days.

disease. It is possible that when serum is administered intraspinally, some additional mechanism comes into play for the disposal of the foreign serum and, therefore, that the interrelations of symptoms, precipitin, and precipitinogen differ somewhat from those found in patients to whom the serum is administered intravenously. Recent

observations by Alexander<sup>4</sup> show that subdural and intravenous injections of the same quantities of foreign serum produce in rabbits distinctly different precipitin curves.

There remains one patient to be considered. This individual was given 630 cc. of antipneumococcus serum intravenously. He had a severe serum reaction; a high titer of precipitin appeared in the circulation synchronous with the cessation of the serum disease, but the precipitinogen, although falling somewhat at the time that precipitin rose to a high concentration, never disappeared entirely from the circulation during the 74 days that he was under observation. This is at variance with the relations which we have found to hold true in the other eighteen patients. There is, however, one consideration which might explain the discrepancy. This patient was one of the first studied, and at this time we had not realized the importance in precipitinogen determination of testing the anti-horse rabbit serum for traces of antigen used in immunizing the rabbit. It is possible, therefore, to explain the discrepancy on the assumption that the precipitating serum used contained traces of antigen and that the precipitin in the patient's serum gave a precipitate with the antigen still present in the anti-horse rabbit serum. This, of course, would make it appear that there was precipitinogen in the circulation of the patient, when it had, perhaps, entirely disappeared. It cannot be proved that this explanation is correct, but it seems plausible.

Summarizing then the results on nineteen patients, we find that eleven of them are distinctly of one type. These are the good precipitin formers who have severe serum disease and get rid of precipitinogen soon after the symptoms have subsided. The second group, contrasting sharply with these, consists of four patients. They are poor precipitin formers, have little or no serum disease, and retain the precipitinogen in the circulation for many weeks and perhaps months. Three of the remaining four patients are more or less distinctly intermediate forms, and the single patient in whom the results appear completely at variance with the observations on the other eighteen may perhaps be explained by a technical error.

<sup>4</sup> Alexander, H. L., *J. Exp. Med.*, 1921, xxxiii, 471.

## DISCUSSION.

It has long been known that individuals show wide variations in their reaction to the administration of foreign serum. At one end of the scale is the individual who is naturally hypersensitive to horse serum. He is often a horse asthmatic with an inherited tendency to hypersensitiveness. He is exquisitely hypersensitive, reacting immediately with violent symptoms to even minute quantities of horse serum. Reactions of this type are sometimes fatal. At the other end of the scale is the individual who does not show a serum reaction after the intravenous administration of even several hundred cubic centimeters of serum. There is clearly wide variation in the response to foreign serum among individuals who at the time that serum is first administered are not demonstrably hypersensitive. Some have severe serum disease, some have only mild symptoms of short duration, and some have no symptoms whatever. That such variations, when large amounts of serum are given, are not dependent upon the amount of serum administered is evident from the results in the present series of patients. The average amount of serum given to the four patients in the group showing no serum disease, or only very mild symptoms was 460 cc.; the average amount given to the patients who had severe serum reactions was 270 cc. When smaller amounts of serum are used, as in diphtheria immunization, and the injections are made subcutaneously or intramuscularly, there appears to be a relation between the incidence of serum reactions and the amount used.<sup>5</sup> With the larger doses, the incidence of reactions is higher, but when amounts over 100 cc. are given, the variations in the intensity and duration of the reaction lead to the conclusion that there is an intrinsic difference between the susceptible and the insusceptible individuals.

What we have found to be characteristic of the susceptible group of patients as far as precipitin, precipitinogen, and symptoms are concerned corroborates the observations of Longcope and Rackemann<sup>1</sup> on the relation of circulating precipitin to the cessation of symptoms, and lends further support to the conception of serum dis-

<sup>5</sup> Daut, M., *Jahrb. Kinderheilk.*, 1897, xliv, 289. Sturtevant, M., *Arch. Int. Med.*, 1916, xvii, 176. Weaver, G. H., *Arch. Int. Med.*, 1909, iii, 485.

ease as an antigen-antibody reaction. Efforts to discover the mechanism of antigen-antibody reactions have resulted in the development of two hypotheses which differ as to the site in which the reaction occurs. One school localizes the reaction within the circulation and holds to the view that the reaction is dependent upon an intravascular union of antigen and antibody. The other school believes that this union takes place chiefly within the tissue cells. No attempt will be made to discuss the voluminous and controversial literature on this question, but it suffices to say that, at present, a preponderance of competent opinion supports the cellular hypothesis. With the facts at present available, the most plausible explanation of the mechanism of these reactions<sup>6</sup> is, therefore, that the foreign serum unites with the tissue cells; that as a result of such union, antibodies are produced; that the intracellular union of antigen and antibody causes the symptoms; and that after a time, the antibody is produced in excess and ceases then to be exclusively intracellular. At this time it becomes demonstrable in the circulation. For a time thereafter, there are both antigen and antibody free in the circulation. If large amounts of antibody reach the circulation, the antigen soon disappears. This disappearance of antigen does not necessarily imply an intravascular union of antigen with antibody, although such a mechanism might account for it. It is also possible that at this time, with greater amounts of intracellular antibody available, the tissue cells appropriate the antigen with greater avidity. All that can be stated at present is that during this period when precipitin is abundant, the foreign serum disappears.

The varying degrees of intensity of reaction by susceptible individuals should therefore be thought of either as varying degrees of susceptibility to a toxic product of intracellular antigen-antibody reaction, or as varying degrees of ability to form antibodies. The latter hypothesis seems to accord better with the facts, because, in general, it is the good precipitin formers who have severe serum reactions. The parallelism is not exact, nor would one expect it to be so, unless there were proof that the precipitin in the circulation is exactly proportional to the intracellular precipitin. And there is no

<sup>6</sup> Weil, R., *J. Immunol.*, 1916-17, ii, 399.

proof of this. A possible explanation of such varying degrees of efficiency with which different individuals form antibodies is discussed below.

In the group of patients found to be insusceptible to serum reactions, in whom little or no precipitin is found, and in whose circulation the foreign serum is present for a long period, there clearly is something which acts as a protective mechanism. Three possibilities perhaps offer plausible explanations for the basis of this failure to produce precipitins and the persistence of the precipitinogen in the circulation. (1) In these individuals there may be something in the circulation which stands as a barrier between the foreign protein in the blood stream and the interior of the cells, preventing the union of foreign protein and tissue cell, and thus interfering with an essential phase of antibody production. (2) The tissue cells of these individuals may be impermeable to the foreign serum. If either one of these conditions existed, little or none of the foreign serum would penetrate the cells, and hence little or no antibody would be formed and serum disease would be mild or absent. (3) In terms of Ehrlich's side-chain theory, the difference between the susceptible and the insusceptible individuals might consist in a deficiency or absence of haptophore groups in the tissue cells of the latter. Under any circumstances, with failure of the cells to take up the foreign protein, it might persist in the circulation until slowly disposed of by some other mechanism.

It is interesting in this connection to recall some observations made years ago by Metchnikoff.<sup>7</sup> In studying the mechanism of natural immunity, he found that a number of species were insusceptible to certain bacterial toxins which were extremely poisonous for other species. Spiders and scorpions were found to be unaffected by tetanus toxin. Nor could antitoxin be demonstrated in the blood of these animals, so the natural immunity could not be ascribed to antitoxic properties of the body fluids. In these animals, however, the injected toxin soon disappeared from the circulation. The immunity of fowls to tetanus toxin was known even before Metchnikoff's studies. The fowl also tolerates large doses of tetanus toxin but can nevertheless be tetanized if it is previously weakened by exposure to cold or if sufficiently large doses are used. That this immunity is not due to the presence or production of antitoxin was shown by Vaillard<sup>8</sup> who

<sup>7</sup> Metchnikoff, E., *Immunity in infective diseases*, translated by Binnie, F. G., Cambridge, 1905.

<sup>8</sup> Vaillard, *Compt. rend. Soc. biol.*, 1891, xliii, 462.

demonstrated that the serum contains no antibody. Moreover, von Behring<sup>9</sup> showed that fowls are highly susceptible if the toxin is injected directly into the brain. Metchnikoff also studied green lizards, marsh turtles, and the larvæ of the rhinoceros beetle (*Oryctes nasicornis*). These animals tolerate, without symptoms, enormous doses of tetanus toxin and not only do not form any antitoxin, but retain the toxin in the circulation for a long period of time, even months.

Phenomena of this type in lower animals suggested to both Metchnikoff<sup>7</sup> and von Behring<sup>10</sup> the possible relation of cell permeability to natural immunity. Our observations on serum disease in man are strikingly analogous to those of Metchnikoff on turtles, lizards, and beetles and suggest anew a factor which may be important in natural immunity.

Investigators in the field of natural immunity have been occupied chiefly in studying different degrees of susceptibility characterizing races or species. On epidemiological grounds alone, it is clear, however, that within any given species or race, there are individual variations in susceptibility to infection for which no adequate explanation exists. From this standpoint, therefore, the demonstration of the differences which we have found to exist between susceptible and insusceptible individuals of the same race may prove to be significant.

#### SUMMARY.

1. Studies on nineteen patients to whom foreign serum had been administered for therapeutic purposes are reported. Analysis of the results obtained by following the precipitin and precipitinogen in the circulation and comparing these factors with the time of appearance, intensity, and duration of the symptoms shows that the nineteen patients fall into three groups.

2. The first group includes eleven patients. These were good precipitin formers, they had severe serum disease, and the precipitinogen disappeared from the circulation near the time that the symptoms subsided.

<sup>9</sup> von Behring, E., in Eulenberg, A., and Samuel, Lehrbuch der allgemeinen Therapie, Berlin and Vienna, 1899, iii, 992.

<sup>10</sup> von Behring, E., in Eulenberg, A., Encyclopädische Jahrbücher der gesamten Heilkunde, Vienna and Berlin, 1900, ix, 203.

3. The second group includes four patients who had little or no serum disease, in whose circulation little or no precipitin was demonstrable, and in whom the precipitinogen persisted in the circulation as long as the patients could be kept under observation—from 52 to 67 days.

4. The remaining four patients form a more or less distinctly intermediate group.

5. The results lend further support to the conception of serum disease as an antigen-antibody reaction.

6. The possibility that our results indicate a factor which may be important in the mechanism of natural immunity is discussed.

We wish to thank Miss Emily Frühbauer for technical assistance.



## STUDIES ON MEASLES.

### III. ACQUIRED IMMUNITY FOLLOWING EXPERIMENTAL MEASLES.

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(Received for publication, February 17, 1921.)

It has been shown in a preceding paper<sup>1</sup> that monkeys (*Macacus rhesus*) inoculated intratracheally with unfiltered or filtered nasopharyngeal washings from patients in the prodromal or early eruptive stage of measles exhibit an illness which closely resembles measles in man in its course and symptomatology. It has furthermore been shown<sup>2</sup> that the lesions which develop in the skin and buccal mucous membrane during the course of the infection in monkeys present essentially the same histologic picture that is found in the corresponding lesions of human measles. The experimental infection has been successfully transmitted<sup>1</sup> from monkey to monkey with the development of the same group of symptoms and pathologic lesions in the passage animals. These fundamental points of similarity between measles in man and the experimental disease in monkeys would appear sufficient to warrant the application of the term "experimental measles" to the latter condition. It has, nevertheless, seemed desirable to determine whether further points of resemblance between the two might not be shown.

Since an apparently permanent immunity against reinfection characteristically follows one attack of measles, the same phenomenon should hold true with respect to the experimental disease if the two conditions are to be regarded as similar. Furthermore, an acquired immunity, if present, should theoretically be efficient against a virus of heterologous source as well as against that of homologous origin, since there is little clinical evidence to show that one attack of measles fails to confer an immunity that is effective against all subsequent

<sup>1</sup> Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 385.

<sup>2</sup> Blake, F. G., and Trask, J. D., Jr., *J. Exp. Med.*, 1921, xxxiii, 413.

exposures. Authentic reports of repeated attacks of measles in the same individual are so few as to be negligible in this connection. In order to test the validity of the foregoing assumptions a series of reinoculation experiments in monkeys which had recovered from a previous attack of experimental measles has been carried out as described below.

#### EXPERIMENTAL.

Six monkeys which had previously been inoculated with nasopharyngeal washings from cases of measles and had recovered from the ensuing attack of the experimental disease<sup>3</sup> have been subjected to reinoculation with material containing the virus of measles (Table I). In five instances virus of heterologous source was used, in one the homologous virus. In two monkeys the material, consisting of the supernatant fluid from an 0.85 per cent salt solution emulsion of the skin and buccal mucosa of a monkey killed on the 4th day of experimental measles, was injected intratracheally. In four monkeys whole blood withdrawn from a monkey on the 3rd day of experimental measles was injected intravenously. The intervals elapsing between recovery from the preceding experimental measles and the time of reinoculation varied from 12 to 254 days. None of the six monkeys following reinoculation showed any evidence of infection with the virus of measles, while the control normal monkeys, inoculated at the same time with equivalent amounts of the same material, developed the characteristic symptoms and pathologic lesions of the experimental disease. The protocols follow.

*Experiment 1.*—June 8, 1920. Monkeys 6, 9, and 19 were injected intratracheally at 12.15 p.m., 12.30 p.m., and 12.45 p.m., respectively, each with 10 cc. of the unfiltered supernatant fluid of an 0.85 per cent salt solution tissue emulsion (Virus MC. 3). The emulsion had been prepared from the minced and ground skin and buccal mucosa of Monkey 16, which was killed on the 4th day of experimental measles, about 24 hours after the first appearance of the exanthem. Monkey 6 had been inoculated Apr. 9, 1920, with pooled, filtered nasopharyngeal washings (Virus AM) from two sisters with measles and had recovered from the ensuing experimental measles on Apr. 23, 46 days before reinoculation. Monkey 9 had been inoculated May 12, 1920, with filtered nasopharyngeal washings (Virus

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<sup>3</sup> For a detailed description of the first attack of experimental measles in these animals see Blake and Trask.<sup>1</sup>

MC) from a patient with measles and had recovered from the ensuing attack of experimental measles on May 27, 1920, 12 days before reinoculation. Monkey 19 was a normal monkey and served as a control.

TABLE I.  
*Immunity Following Experimental Measles.*

Monkey No.	First inoculation.			Second inoculation.			
	Date.	Virus.	Result.	Date.	Interval after recovery from first attack.	Virus.	Result.
	1920			1920	days		
6	Apr. 9	AM	Experimental measles Apr. 16-22.	June 8	46	MC.3*	Remained well.
9	May 12	MC	Experimental measles May 20-26.	" 8	12	" 3	" "
19 (con- trol).				" 8		" 3	Experimental measles, June 14-17. Killed.
2	Mar. 24	RG	Experimental measles Mar. 29-Apr. 4.	Dec. 15	254	JJ. 5	Remained well.
3	" 24	RK	Experimental measles Apr. 3-10.	" 15	248	" 5	" "
5	Apr. 9	AM	Experimental measles Apr. 16-22.	" 15	236	" 5	" "
8	May 12	MC	Experimental measles May 19-27.	" 15	201	" 5	" "
46 (con- trol).				" 15		" 5	Experimental measles Dec. 18-27.

\* The figure indicates the number of monkeys through which the virus had been passed.

Monkeys 6 and 9 showed no evidence of measles during 21 days observation. There were no conjunctivitis, no enanthem, and no exanthem, the eyes, buccal mucosa, and skin remaining normal in appearance throughout this period. Monkey 19 after an incubation period of 6 days developed the characteristic symptoms

of the experimental disease. On the 7th day a few discrete hyperemic spots appeared on the labial mucous membranes. On the 8th day the animal was listless; the conjunctivæ were injected; fresh Koplik spots had appeared on the mucous membrane of the cheeks. On the 9th day there was a well developed, confluent enanthem on the mucous membrane of the lips, gums, and cheeks. On the 10th day a few discrete, red maculopapules appeared about the lips, on the chin, and behind the ears. The animal was killed and the infection was successfully transmitted to two other monkeys. Histologic sections of the labial mucosa and tongue show the typical lesions of measles. The endothelial cells of the capillary walls are greatly swollen. There are a marked accumulation of endothelial leucocytes and some serous exudate about the capillaries, especially in the papillæ. In the stratified epithelium of the labial mucosa are many small foci (Koplik spots) showing endothelial leucocytes, serous exudate, and beginning necrosis of the epithelial cells. In some of these the process is more advanced and there is maceration of the epithelium, with shallow ulceration and secondary invasion by polymorphonuclear leucocytes. A few similar foci are seen in the epithelium of the tongue. There is also a more diffuse infiltration of the epithelium by endothelial leucocytes.

*Experiment 2.*—Dec. 15, 1920. Monkeys 2, 3, 5, 8, and 46 were injected intravenously in turn, each with 5 cc. of citrated whole blood (Virus JJ. 5) withdrawn from Monkey 36 on the 3rd day of experimental measles about 6 hours after the first appearance of the exanthem.

Monkey 2 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RG) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 5, 254 days before reinoculation.

Monkey 3 had been inoculated Mar. 24, 1920, with nasopharyngeal washings (Virus RK) from a patient with measles and had recovered from the ensuing attack of experimental measles on Apr. 11, 248 days before reinoculation.

Monkey 5 had been inoculated Apr. 9, 1920, with pooled nasopharyngeal washings (Virus AM) from two cases of measles and had recovered from the ensuing attack of experimental measles on Apr. 23, 236 days before reinoculation.

Monkey 8 had been inoculated May 12, 1920, with nasopharyngeal washings (Virus MC) from a case of measles and had recovered from the ensuing attack of experimental measles on May 28, 201 days before reinoculation.

Monkey 46, normal, served as a control.

Monkeys 2, 3, 5, and 8 showed no evidence of infection during 21 days observation. They were well and active throughout this period. There were no conjunctivitis, no enanthem, and no exanthem. Monkey 46, after an incubation period of 3 days, developed the characteristic symptoms of measles. On the 4th day three Koplik spots appeared on the mucous membrane of the upper lip. On the 5th day the conjunctivæ were injected. On the 6th day a cluster of fresh Koplik spots was present on the mucous membrane of the lower lip. The animal was drowsy and listless. On the 7th day there were confluent patches of hyperemic enanthem studded with minute white specks on the labial mucosa. A few

red maculopapules appeared on the lower abdomen and inner surfaces of the thighs. By the 10th day there was a thick, red, maculopapular exanthem on the face, neck, chest, abdomen, and legs. By the 12th day the exanthem had faded; the exanthem was fading and showed fine branny desquamation. By the 14th day the animal had recovered except for slight remaining desquamation. Blood cultures on the 5th, 6th, and 7th days showed no growth. The infection was successfully transmitted from this animal to another monkey by means of blood withdrawn on the 5th, 6th, and 7th days. A section of skin excised<sup>4</sup> from the thigh on the 10th day shows the characteristic histologic picture of measles. About the capillaries and small veins in the upper layers of the corium there is a marked accumulation of endothelial leucocytes. Occasionally one is seen in mitosis. A few polymorphonuclear leucocytes are also present. Focal accumulations of endothelial leucocytes with vacuolation and necrosis of epithelial cells are seen in the epithelium of many of the hair sheaths and sebaceous glands. The epidermis shows vacuolation and necrosis of the cells of the Malpighian layer in minute foci. These areas are invaded by endothelial leucocytes. In the cornified layer are occasional, small, deeply staining plaques with the remains of minute vesicles beneath them.

#### DISCUSSION.

The result of the foregoing experiments shows that one attack of experimental measles confers an apparently complete immunity against reinfection with measles for at least a considerable period. In all probability this immunity is permanent. In Experiment 1 it should be noted that Monkeys 6 and 9 were originally inoculated with filtered (Berkfeld N) nasopharyngeal washings. Their subsequent immunity, therefore, not only provides additional evidence of the similarity between human measles and the experimental disease but also tends to confirm the filterable nature of the virus. The strain of virus used in the reinoculation of Monkey 6 was of different origin from the strain with which this animal was originally inoculated, while with Monkey 9 the same strain of virus was employed in both the first and second inoculations. Since there was no apparent difference in the immunity of the two animals it would seem probable that the immunity provided by one attack of experimental measles is as efficient against a heterologous virus as against the homologous one. This is further supported by the result of the second experiment, in which four monkeys originally inoculated with strains of virus from four different sources exhibited a complete immunity against reinfection with a virus obtained from a still different source.

<sup>4</sup> This was done under ether anesthesia.

This result, as has been pointed out, was to be expected. Furthermore, it would suggest the probability that all strains of measles virus are of homologous nature in as far as their property of stimulating immunity is concerned, a fact which, of course, might readily be predicated from clinical observation.

The results of the two experiments, although they do not provide an explanation of the mechanism of acquired immunity against measles, nevertheless suggest certain possibilities. The course of measles, itself, in conjunction with evidence already presented<sup>1</sup> concerning the infectivity of the blood in the experimental disease, leaves little reason to doubt that the virus gains access to the blood by way of the respiratory mucous membrane and is subsequently distributed by the blood stream to the skin and buccal mucosa where it sets up the characteristic lesions of the disease. It is conceivable that the process of immunity against reinfection might reside in the respiratory mucous membrane which, in the immune animal, would present a barrier to invasion of the body by measles virus. While this supposition might serve as a possible explanation of the immunity exhibited by Monkeys 6 and 9 which were reinoculated by the intratracheal route, it is obviously inadequate in the case of the second experiment in which all the monkeys were inoculated intravenously. Since these animals showed an apparently complete immunity, it is clear that the immunity is not solely, if at all, dependent upon a possible barrier offered by the respiratory mucous membrane of the immune animal. That it is a function of the body tissues or fluids would seem more probable. Whether the immunity is humoral or cellular or both, however, only further experiment can determine.

#### SUMMARY.

It is shown that monkeys which have recovered from experimental measles are immune to reinfection with the virus of measles irrespective of whether the virus is of homologous or heterologous origin. In this respect experimental measles in the monkey corresponds with measles as observed in human beings, and the result is the same whether the virus is inoculated on the respiratory mucous membrane or is injected intravenously.

#### CONCLUSION.

Experimental measles in the monkey, like measles in man, is followed by an acquired immunity against the disease.

# STUDIES ON BLOOD CHANGES IN PNEUMOCOCCUS INFECTIONS.

## AN EXPERIMENTAL STUDY OF THE FORMATION AND FATE OF METHHEMOGLOBIN IN THE BLOOD.

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(Received for publication, January 27, 1921.)

### INTRODUCTION.

#### *Methemoglobin Formation in Pneumonia.*

The pneumococcus, both *in vitro* (Gilbert and Fournier, 1896; Grüter, 1909; and Peabody, 1913) and *in vivo* (Butterfield and Peabody, 1913), has been shown to transform hemoglobin into methemoglobin. In consequence it seemed that methemoglobin formation might be the chief cause of the cyanosis sometimes observed in pneumonia. A study of the oxygen content and capacity of venous and arterial blood, however (Stadie, 1919), showed that the great and constant abnormality accompanying cyanosis was an increased proportion of reduced hemoglobin to oxyhemoglobin in the arterial blood. Therefore the essential cause of the cyanosis of pneumonia is incomplete oxygenation of the arterial blood, rather than the presence of methemoglobin in the blood. In only one of the thirty-two cases studied by the author was the oxygen capacity significantly reduced, as it would be if any considerable portion of the hemoglobin were changed to methemoglobin. On the contrary, in many cases the oxygen capacity of the blood was above the normal average.

Although we had but one case in which the oxygen capacity fell significantly, Peabody (1913) and Harrop (1919) have observed several cases in which such a fall occurred. Of eleven cases Peabody observed a marked fall of oxygen capacity in three, and Harrop in nine cases found a decrease of one-half in the total oxygen capacity in two. All these cases had positive blood cultures. In two other cases of Harrop's with negative blood cultures there was a decrease in oxygen capacity, but of less degree and over a greater period of time. It is possible that in these cases a considerable proportion of the hemoglobin may be altered into methemoglobin, and even in patients that do not show definitely lowered oxygen capacity small amounts of methemoglobin may be formed, and either be eliminated from the blood, or remain in it. In order to obtain more complete

evidence on these points a quantitative method for determination of methemoglobin was devised (Stadie, 1920), and in the present study it has been utilized in experimental work aimed to obtain evidence on the points in question.

#### *General Conditions for Methemoglobin Formation.*

The agents which change hemoglobin to methemoglobin are of varied nature. They include the following groups of known chemical substances: oxidizing substances, ozone, iodine, chlorates, ferricyanides, nitrites, nitrates, and azo compounds; reducing substances, pyrogallol, hydroquinone, hydroxylamine, etc.; organic bases, aniline, phenacetin, acetanilide, and toluidine; salts, sodium chloride in concentration above 1.5 per cent and ammonium sulfate in saturated solution. They also include several races of bacteria: *Streptococcus viridans*, cholera, pneumococcus, Gaertner bacillus, and certain nitrosobacilli (Wallis, 1913-14).

*In vitro* all these agencies produce methemoglobin with greater or less facility. Potassium ferricyanide rapidly forms methemoglobin from oxyhemoglobin. An amount of oxygen is liberated which is equivalent to the dissociable oxygen originally combined as oxyhemoglobin. Sodium nitrite converts hemoglobin to methemoglobin *in vitro*, but more slowly than potassium ferricyanide. The nitrite liberates an amount of oxygen equivalent to the amount necessary to change the nitrite to nitrate (Barcroft and Müller, 1911-12). In other words, two molecules of nitrite transform one molecule of hemoglobin, producing one molecule of oxygen and one molecule of methemoglobin. Hydroxylamine transforms hemoglobin quantitatively, molecule for molecule (Letsche, 1912).

Of the above agents, we have used in our experiments potassium ferricyanide and sodium nitrite.

#### *Characteristics of Clinical and Experimental Methemoglobinemia.*

*Agents.*—Human cases of methemoglobinemia are the result of poisoning with various agents, of which aniline is perhaps the most familiar. In laboratory animals methemoglobin is produced easily by nitrites, potassium ferricyanide, acetanilide, and the pneumococcus.

*Symptoms.*—In the overwhelming methemoglobinemia produced experimentally it is difficult to dissociate the symptoms produced by



the drug from those produced by the methemoglobin. Rapid breathing and air-hunger are constant symptoms in severe grades of methemoglobinemia, and when 70 to 80 per cent of the hemoglobin is changed the animal exhibits all the signs of acute suffocation and dies in a short time. In milder instances with a destruction of 25 to 50 per cent of the hemoglobin, usually there are no symptoms in rabbits. It is doubtful whether the mere presence of large amounts of methemoglobin in the body is harmful.

Cyanosis is a regular accompaniment of methemoglobinemia. This, of course, is due to the fact that the presence of methemoglobin in blood gives it a dark color closely resembling that of venous blood. Transformation of as little as 5 per cent of the hemoglobin to methemoglobin in blood gives it a dark color. The blood does not become bright red on exposure to the air and is easily distinguishable from normal oxygenated blood. The cyanosis itself is indistinguishable from the cyanosis due to oxygen unsaturation of the arterial blood. Two important differences, however, may be noted: (1) the cyanosis of oxygen unsaturation usually accompanies pulmonary or heart disease and varies in intensity with change of position, coughing, and exertion; (2) the administration of oxygen may diminish this cyanosis. The final differentiation is made by the spectroscope or by methemoglobin determination, for which a method has been recently published (Stadie, 1920).

*Pathology.*—No characteristic changes of the organs have been described in animals in which a severe methemoglobinemia has been induced. Certain hemorrhagic changes and areas of necrosis in the liver and spleen have been described, but these have been inconstant and have followed large doses of potassium chlorate, and consequently might easily have been due to this substance rather than to the methemoglobin.

The proportion of hemoglobin changed may be as great as 100 per cent, as after the intravenous injection of sodium nitrite in rabbits. With acetanilide or sodium nitrite in proper doses a reduction in the amount of hemoglobin of 60 to 70 per cent may be brought about easily. The animals often recover; the sudden flooding of the body by so large an amount of methemoglobin is without apparent permanent effect.

Methemoglobin may exist in the blood in two distinct conditions. First, it may be present in the plasma alone, a true methemoglobinemia. This is, however, rare, but is illustrated by Brandenburg's case of potassium chlorate poisoning which showed a rapid decrease of red blood cells from 4,300,000 to 1,600,000 in 6 days. The serum showed methemoglobin spectroscopically. Second, the methemoglobin is present within the red blood cells—a condition of methemoglobinemia. This is the usual occurrence. Cases of nitrite, acetanilide, and nitrobenzene poisoning and bacterial infections with methemoglobin formers fall into this class.

#### EXPERIMENTAL.

*Fate of Methemoglobin.*—Methemoglobin solutions injected intravenously are rapidly eliminated from the blood. Table I shows

TABLE I.

*Elimination of Methemoglobin Following Intravenous Injection in Rabbits.*

Rabbit No.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin spectrum.		
				Serum.	Cells.	Urine.
1	<i>p.m.</i>		<i>gm.</i>			
	12.10		13.87			
	12.20	2 gm. of methemoglobin intravenously.				
	12.45		12.04	Negative.	Negative.	
	4.00	(Killed.)*	11.87	"	"	++++
2	<i>a.m.</i>					
	9.05		10.03			
	11.00	1.7 gm. of methemoglobin intravenously.				
	11.15		10.03	Negative.	Negative.	++++

\* Extracts of lungs, liver, spleen, heart, feces, and intestines showed no methemoglobin spectroscopically.

the extreme rapidity with which methemoglobin dissolved in the plasma disappears. The hemoglobin was determined gasometrically by Van Slyke's method (1918), the methemoglobin by the author's method (1920).

Within 15 to 25 minutes an amount of methemoglobin equal to 20 to 22 per cent of the total hemoglobin was completely removed from

the blood so that none could be found in it spectroscopically. At least part of the methemoglobin was excreted in the urine.

*Storage of Methemoglobin.*—Extracts of the various organs made immediately post mortem revealed no evidence that methemoglobin is accumulated in any one place. When the methemoglobin is present in the red blood cells only, even when all the hemoglobin is changed to methemoglobin, with death by virtual suffocation due to lack of labile oxygen, the plasma is always free from methemoglobin bands.

The methemoglobin produced in the red blood cells is rapidly destroyed. In the milder cases of experimental methemoglobin-cythemia this destruction was found to be so rapid that repeated and careful examinations of blood cells, plasma, urine, and tissues failed to reveal its presence.

Table II gives the results from rabbits injected with solutions of potassium ferricyanide. The action of this substance *in vivo* is relatively slow, but by repeated injections over a period of several hours almost 50 per cent of the hemoglobin may be changed. In the three rabbits the potassium ferricyanide changed 43, 30, and 19 per cent respectively of the hemoglobin, doubtless into methemoglobin, and yet the latter was so rapidly destroyed that practically none of it could be found in the blood.

In another rabbit (Table III) within 5 days the hemoglobin diminished 39 per cent, yet no methemoglobin was found spectroscopically in either plasma or cells. Moreover, the urine and the aqueous extracts of liver, lungs, spleen, kidney, heart, skeletal muscle, intestines, and feces showed no methemoglobin by spectroscope.

The rapid production of methemoglobin, as by the injection of sodium nitrite, by which an easily controllable degree of methemoglobin formation may be brought about, gives the same results.

In Rabbit 7 (Table IV) practically all the hemoglobin was changed to methemoglobin, the animal dying immediately of suffocation. The pigment is wholly in the cells. Rabbit 8 lived for 26 minutes with hemoglobin of only 2.9 gm. per 100 cc. of blood, or 23 per cent of the initial value. Yet within this short time the methemoglobin had decreased to 6.7 gm. per 100 cc. of blood. A rabbit of 2.85 kilos has 142 cc. of blood (5 per cent by weight (Van Slyke and Salvesen, 1919) ), or in this case 18.1 gm. of hemoglobin, of which at death 4.1

TABLE II.

*Production of Methemoglobin in Rabbits by Potassium Ferricyanide.*

Rabbit No.	Date.	Time.	Injection.	Hemo- globin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.
	1919	a.m.		gm.	gm.
3	Apr. 25	9.30		15.2	
		10.15	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		10.30		13.2	
		11.50	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
	Apr. 29 May 6	12.15		11.0	
		2.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		3.15		8.8	
				8.9	
				8.7	0.0
4	May 7	a.m.			
		11.35		11.1	
		11.45	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		p.m.			
		12.45		10.5	0.0
		1.02	5 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.15		10.0	0.3
		2.30	5 cc. of 0.02 M potassium ferricyanide intravenously.		
5	May 9	2.31	(Animal died.)	7.8	0.2
		a.m.			
		9.20		13.3	
		9.40	20 cc. of 0.02 M potassium ferricyanide intravenously.		
		9.45		14.1	0.0
		10.30		13.0	0.4
		11.45		12.5	0.0
		p.m.			
		2.07	10 cc. of 0.02 M potassium ferricyanide intravenously.		
		2.30		10.8	0.0

TABLE III.  
*Production of Methemoglobin in Rabbits by Potassium Ferricyanide.*

Rabbit No.	Date.	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemoglobin per 100 cc. of blood.	Methemoglobin spectrum.	
						Plasma.	Cells.
6	1910 June 5	m.					
		12.00					
		p.m.					
	June 6	2.27					
		a.m.					
		10.00					
		11.07					
		11.47					
		p.m.					
		12.21					
	June 7	1.45					
		2.47					
		4.00					
		a.m.					
		10.58					
		11.45					
	June 9	p.m.					
		1.15					
		3.40					
June 11		4.32					
		4.30					

\* Colorimetric determination.

† Extract of liver, lungs, spleen, heart, skeletal muscle, intestines, and feces showed no methemoglobin spectrum. Urine negative also.

gm. were left. Of the 14 gm. of methemoglobin formed, only 9.5 gm. remained at death. In other words, 4.4 gm. of methemoglobin were destroyed in 26 minutes. In Rabbit 9, 1.8 gm. of methemoglobin were destroyed in 30 minutes. In all these rabbits no methemoglobin was found in the plasma, although the blood was dark chocolate in color; it was present in the cells only.

Therefore even in extreme cases of methemoglobin production in which death results quickly from an insufficient oxygen supply, there is a rapid destruction of methemoglobin. When the methemoglobin is produced more slowly this destruction is fast enough to prevent its accumulating in the blood in sufficient quantity to be detected by the spectroscope. Not only is this true when chemicals are the causative agent, but also when methemoglobin-producing bacteria act *in vivo*.

However, when the production of methemoglobin is very extensive, e. g. 30 to 50 per cent of total pigment, and sudden, as following intravenous injections of sodium nitrite, methemoglobin may be found in the blood, but always in the red blood cells. Even in these instances, if the animal survives for a comparatively brief time, the methemoglobin disappears from the blood.

The mechanism of this disappearance has not been determined. Pearce, Austin, and Eisenbrey (1912) studied the fate of hemoglobin injected intravenously and found that no hemoglobin was eliminated through the kidney unless the rate of injection was above a minimum which was high, but that nevertheless the injected hemoglobin rapidly disappeared from the blood stream. Furthermore, Whipple and Hooper (1913) showed that injected hemoglobin is changed rapidly to bile pigments in normal dogs and in dogs with Eck fistula or hepatic ligation. Since methemoglobin is closely related chemically to hemoglobin it is possible that it is similarly disposed of.

*Action of Pneumococci on Hemoglobin in Vivo.*—Grüter (1909) inoculated a cat intraperitoneally with a large amount of pneumococcus culture but was unable to demonstrate methemoglobin in the blood. Peabody (1913) developed the subject further by inoculating rabbits intravenously with the organisms from 300 to 600 cc. of 24 hour broth cultures of pneumococci. Death resulted within a few hours and the blood always showed on direct film enormous num-

TABLE IV.  
*Intravenous Injection of Sodium Nitrite in Rabbits. Rapid Formation and Disappearance of Methemoglobin.*

Rabbit. No.	Weight. <i>kg.</i>	Time.	Injection.	Hemoglobin per 100 cc. of blood.	Methemo- globin per 100 cc. of blood.	Methemo- globin* destroyed.	Methemoglobin spectrum.		
							Plasma.	Cells.	Urine.
7		<i>a. m.</i> 9.15	0.95 gm. of sodium nitrite intra- venously. (Died.)	7.8		<i>gm.</i>			
		9.30							
		9.31					Negative.	+++	Negative.
8	2.85	<i>p. m.</i> 3.00	0.1 gm. of sodium nitrite per kilo intravenously. (Died.)	12.7	0.0	<i>gm.</i>			
		3.21							
		3.47					Negative.	+++	Negative.
9	1.7	<i>a. m.</i> 9.00	0.12 gm. of sodium nitrite per kilo intravenously. (Died.)	10.0	0.0	<i>gm.</i>			
		9.30							
		10.00					Negative.	+++	Negative.

\* Estimated on the assumption that the blood volume is 5 cc. per 100 gm. of body weight (Van Slyke and Salvesen, 1919).

bers of pneumococci. With these overwhelming pneumococcemias Peabody showed that there was a rapid fall in total hemoglobin as measured by oxygen capacity, but he was rarely able to demonstrate the presence of methemoglobin in the blood. He nevertheless concluded that this fall in total oxygen capacity (hemoglobin) was due to a methemoglobin production. However, when these experiments are repeated with quantitative determination of methemoglobin, it is clear that bacterial methemoglobinemia is similar to that produced chemically in that the methemoglobin formed rapidly disappears.

Rabbits were inoculated by ear vein with the centrifuged pneumococci from 24 hour cultures resuspended in a small amount of saline solution. Death occurred in all cases and all showed by direct film of the blood enormous numbers of organisms. The results are shown in Table V.

In Rabbit 10, after 5 hours, despite a fall in hemoglobin of 3.94 gm. per 100 cc. of blood (30 per cent of the total), there was no methemoglobin in the blood. In No. 12, 3.82 gm. of hemoglobin per 100 cc. of blood (30 per cent of the total) were changed, but only 0.83 gm. of methemoglobin per 100 cc. was found. In Nos. 10 and 11 at death considerable amounts of methemoglobin were found in the blood, but amounts representing only 30 to 32 per cent of the total change. It is clear then that the mechanism here is the same that is described in the production of methemoglobin by chemicals. In the gradual change of hemoglobin to methemoglobin the latter pigment disappears as fast as it is formed, so that it is never present in the blood in sufficient concentration to be detected spectroscopically. Only near death does the rapid production of methemoglobin by the huge numbers of pneumococci lead to its accumulation in the blood in quantities greater than the now disordered metabolism can handle. In other words, it is rarely possible to find methemoglobin in the blood, unless just before death, even after considerable changes of hemoglobin to methemoglobin.

It is to be further noted that the methemoglobin present in these instances is entirely intracellular. Examination of the plasma spectroscopically gives negative results, but the centrifuged cells in the last samples of Nos. 11 and 12 contain abundant methemoglobin. The urine also is always negative. The condition is a methemoglobinemia.



TABLE V.

*Production of Methemoglobin by Pneumococci in Rabbits.*

Rabbit No.	Date.	Time.	Conditions.	Hemo-globin per 100 cc. of blood.	Hemo-globin decrease per 100 cc. of blood.	Methemo-globin per 100 cc. of blood.	Remarks.
	1919	a.m.		gm.	gm.	gm.	
10	June 15	11.45		12.98	0.0	0.0	
		p.m.					
		1.00	Inoculated.				
		3.30		10.82	2.16	0.30	Blood bright red.
		5.00		9.04	3.94	0.00	" " "
		8.45	Very sick.	6.02	6.96	2.09	" chocolate-colored.
		9.00	Died.				
		a.m.					
11	June 25	9.00		12.78		0.0	
		10.25	Inoculated.				
	June 26	10.25		11.60	1.18	0.18	Blood bright red.
		p.m.					
		4.19	Died.	5.75	7.03	2.27	" chocolate-colored.
		a.m.					
12	June 30	9.00		12.87		0.0	
		10.50	Inoculated.				
		p.m.					
		3.30	Died.	9.05	3.82	0.83	Blood bright red.

Rabbit 10 received *Pneumococci* Type I from 250 cc. of broth cultures. Rabbit 11 received *Pneumococci* Type I from 100 cc. of broth cultures. Rabbit 12 received *Pneumococci* Type II from 150 cc. of broth cultures. Postmortem films of blood from all the rabbits showed enormous numbers of pneumococci.

#### *Non-Production of Methemoglobin by Pneumococcus Autolysates.*

Grüter (1909) observed methemoglobin formation in the presence of dead cultures, filtrates, and centrifugates. Butterfield and Peabody (1913) also have produced methemoglobin by the autolysates of pneumococcus cultures. Rieke (1904), however, attributed methemoglobin formation to the living pneumococcus and streptococcus only. Cole (1914) likewise reached the same conclusion, since he was unable to demonstrate the production of methemoglobin by filtrates and extracts of pneumococcus cultures.

We have also been unable to show a transformation of hemoglobin to methemoglobin without the living organism. A broth culture of pneumococcus was used in which, after 5 days incubation, all organisms were autolyzed as shown by sterile subcultures. Prepared in this way the pneumococcus autolysate failed to produce methemoglobin when mixed with hemoglobin solutions, as shown in Table VI.

It is quite possible, however, that if, as has been suggested by Avery and Cullen,<sup>1</sup> the proper conditions are found for the reaction or if concentrated solutions of intracellular products obtained by the disintegration of the pneumococcus are used, methemoglobin formation without the living organism may be demonstrated.

TABLE VI.

*Action of Autolysate of Pneumococci on Hemoglobin.*

Tube No.	Conditions.	Broth.	Autolysate.	Hemoglobin solution.	Initial hemoglobin.	Hemoglobin after 20 hrs.	Methemoglobin after 20 hrs.
		cc.	cc.	cc.	per cent	per cent	per cent
1	Room temperature.....	0	10	10	7.14	6.45	0.69
2	Ice box.....	0	10	10	7.14	7.14	0.00
3	Room temperature.....	10		10	7.30	6.67	0.63

## DISCUSSION.

*Methemoglobin Formation in Pneumonia.*—Peabody (1913) was the first to explain fall of blood oxygen capacity observed in some (usually fatal) pneumonia cases as due to a change of hemoglobin to methemoglobin. He suggested that this alteration could take place through the action of a soluble product of bacterial metabolism. If the production of methemoglobin by soluble bacterial products, as suggested by Peabody, is substantiated by further experiments, then it is possible that the decrease in hemoglobin may be brought about by the escape of these products into the blood stream from a focus of pneumococcal infection in the lungs. However, all the cases studied by Peabody (1913) and Harrop (1919) with a sudden and well defined decrease of hemoglobin had a pneumococcemia of profound degree (from 2,500 to 16,000 colonies per cc. of blood). It is, therefore,

<sup>1</sup> Avery, O. T., and Cullen, G. E., unpublished work.

probable that a great production of methemoglobin occurs only when the opportunity for the direct action of the pneumococci on the blood is greatest; *i. e.*, in cases with a bacteremia.

*Methemoglobin and Cyanosis in Pneumonia.*—Although there has been a prevalent belief that methemoglobinemia is a factor in the cyanosis of pneumonia, many observers (Abrahams, Hallows, and French, 1919; Synnott and Clark, 1918; and Peabody, 1913) have looked for it spectroscopically in cases of pneumonia deeply cyanotic, but have failed to find it.

The absence of methemoglobinemia in pneumonia, even when there is a marked fall in oxygen capacity, is, we believe, explainable by the results reported in this paper. Methemoglobin disappears from the circulation with great rapidity, whether it is introduced by injection of methemoglobin or formed within the circulation by the action of chemicals or of pneumococci. If we take into consideration this fact, the ability of the pneumococcus to form methemoglobin, and the consistent failure to find it in the blood in the cyanosis of pneumonia, it appears that the following conclusions represent the most probable explanation of what occurs.

#### CONCLUSIONS.

In the occasional cases of pneumonia which show a decrease in the oxygen capacity of the blood, the decrease is probably due to a formation of methemoglobin. The latter is removed from the circulation, however, as rapidly as it is formed, so that it can seldom be detected even qualitatively, and is probably never a cause of cyanosis.

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# FIBRIN AND SERUM AS A CULTURE MEDIUM.

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(Received for publication, February 17, 1921.)

## INTRODUCTION.

Various artificial media have been used so far in the cultivation of tissues. Lewis and Lewis used bouillon and agar,<sup>1</sup> salt solutions,<sup>2</sup> and other media of known chemical constitution,<sup>3</sup> but the growth was by no means equal to that in plasma or lymph, either in extent or duration. Swezy<sup>4</sup> obtained cell proliferation of chick embryo heart tissue in a medium composed of egg albumin and muscle extract. Carrel and Burrows<sup>5</sup> found homogenic as well as heterogenic serum a useful culture medium for embryonic chick tissue. Ingebrigtsen<sup>6</sup> studied the growth of tissue outside the organism in a medium composed of agar and serum. Smyth<sup>7</sup> devised a so called simplified medium composed of agar and trypsinized peptone solution for embryonic tissue culture. No attempt was made to undertake a comparative quantitative study of the amount of tissue produced in these different media. Burrows<sup>8</sup> pointed out that embryonic tissues grew as well in saline solution as in plasma. He thought that growth consisted in a spreading of the cells and not in any increase in the mass of tissue, and that it appeared to take place at the expense of the original fragment. It is certain that the tissues did not increase

<sup>1</sup> Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

<sup>2</sup> Lewis, M. R., *Anat. Rec.*, 1915-16, x, 287. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1911, lvi, 1795.

<sup>3</sup> Lewis, W. H., and Lewis, M. R., *Anat. Rec.*, 1912, vi, 207.

<sup>4</sup> Swezy, O., *Biol. Bull.*, 1915, xxviii, 47.

<sup>5</sup> Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.

<sup>6</sup> Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397-398.

<sup>7</sup> Smyth, H. F., *J. Med. Research*, 1914-15, xxxi, 255.

<sup>8</sup> Burrows, M. T., *Tr. Cong. Am. Phys. and Surg.*, 1913, ix, 77.

in mass in any of the artificial media, nor could they be kept alive after a certain time. Therefore, these media could not be used for a quantitative study of the problem of growth.

It is known that the presence of embryo juice in adult plasma allows an indefinite growth of the fibroblasts and an increase in the mass of tissue. But the composition of this medium is complex and cannot be modified easily. It would be useful to find a medium endowed with the same properties as plasma and embryo juice, and more adaptable to the nature of the experiment.

The purpose of this article is to describe a technique for preparing a medium composed of fibrinogen, serum, and tissue juice, and to compare the growth of fibroblasts in this medium with that obtained in a medium composed of plasma and embryo juice.

#### EXPERIMENTAL.

The technique of Mellanby<sup>9</sup> was used in the preparation of fibrinogen. 10 cc. of normal adult chicken plasma were diluted with 90 cc. of sterile distilled water, and thoroughly shaken in an Erlenmeyer flask; 1 cc. of a 1 per cent acetic acid solution was added, drop by drop, and at the same time the mixture was agitated. The precipitate was allowed to settle partially in the cold for about 1 hour. The contents of the flask were then shaken and poured into centrifuge tubes, 25 cc. in each tube. After 10 minutes centrifugation, the supernatant fluid was decanted. The tubes were inverted over a sterile piece of filter paper for complete drainage. The precipitate contained in each centrifuge tube was combined and made up to 2.5 cc. with sterile, distilled water. When thoroughly mixed, it had the appearance of rich milk; on standing, a heavy sediment settled, superimposed by a layer of turbid fluid. Equal volumes of this suspension and Ringer's solution formed a slightly hazy, firm homogeneous clot after about 4 minutes. With an equal volume of serum, the mixture formed a clear fluid which did not coagulate after 10 minutes, but on the addition of a trace of embryonic tissue juice, coagulation occurred rapidly. The hydrogen ion concentration of such a preparation of fibrinogen was between 6 and 6.3. A mixture

<sup>9</sup> Mellanby, J., *J. Physiol.*, 1917, li, 396.

of 12.5 per cent fibrinogen suspension, 37.5 per cent chicken serum, and 50 per cent embryonic tissue juice, had a pH of between 7 and 7.3. Such a preparation coagulated in about 1 minute.

The experiments were begun with 48 hour cultures, derived from a strain of connective tissue in its 9th year *in vitro*.<sup>10</sup> Each fragment of tissue was divided in two parts and washed in Ringer's solution for about 40 seconds. One fragment was then cultivated in equal volumes of normal chicken plasma and embryonic tissue juice. The other fragment was cultivated in the experimental medium composed of one-fourth volume of fibrinogen suspension, three-fourths volume of chicken serum, and one volume of embryonic tissue juice. The constituents of this medium were first mixed by drawing them up

TABLE I.

Experiment No.	Culture No.	First passage.			Second passage.			Third passage.			Fourth passage.		
		Relative increase.		Ratio. $\frac{E}{C}$ .	Relative increase.		Ratio. $\frac{E}{C}$ .	Relative increase.		Ratio. $\frac{E}{C}$ .	Relative increase.		Ratio. $\frac{E}{C}$ .
		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.	
1	17555	17.5	16.2	0.93	12.5	11.6	0.93	10.3	9.2	0.89	11.0	8.9	0.81
											9.7	8.0	0.83
2	17536	21.5	18.3	0.85	21.7	19.3	0.88	10.5	8.0	0.76	10.0	9.2	0.92
					21.0	14.6	0.70	10.7	8.8	0.82	8.4	Liquefied.	

and expelling them from a bulb pipette, after they had been dropped into the hollow of a deep, concave slide. After 48 hours the tissues were washed and cultivated in the same medium.

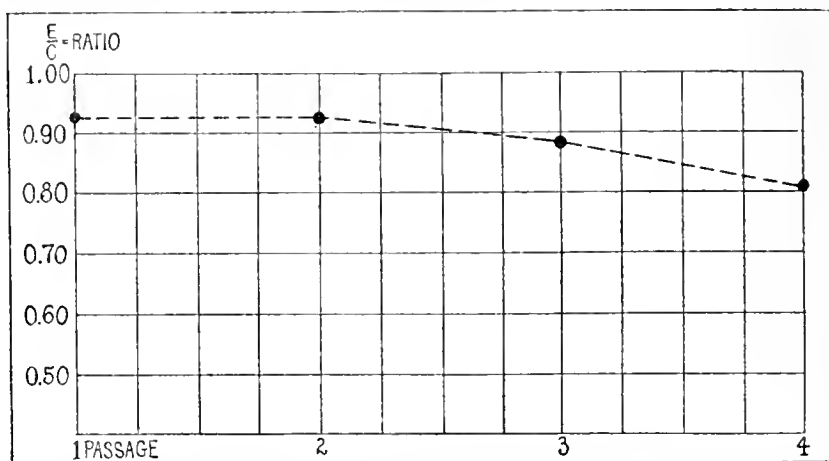
The cultures were incubated at 39°C. Observations were made on the coagulation time, consistency, duration of the coagulum, and character of growth as compared with the controls. The cultures were traced, measured, and the relative increase was calculated, according to the technique mentioned in a previous article.<sup>11</sup>

The technique was developed in the course of thirty-five experiments. The results do not require detailed description because the

<sup>10</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

<sup>11</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 533-534.

appearance of the coagulum and of the growth of the strain of fibroblasts was about the same as that of the control in plasma and embryo juice. Generally, the width of the zone of new tissue and its density were slightly less in the experiment than in the control; the difference in most instances was approximately 10 per cent. In other experiments the tissues were allowed to grow for 48 hours several times, and then transplanted into a medium of the same composition. The results of two of these experiments are given in Table I and in Text-figs. 1 and 2. In the table the figures for the relative growth of the tissue obtained in the control and experiment represent the amount



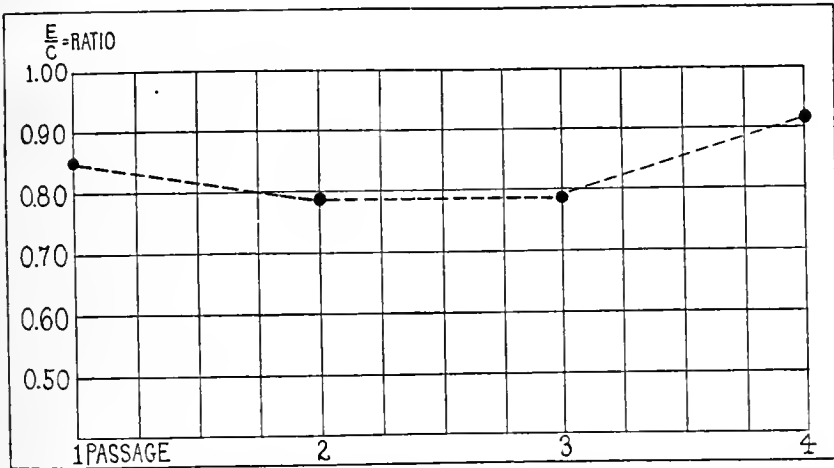
TEXT-FIG. 1. Experiment 1. Ratio between the two relative growths, experiment and control.

of growth which was obtained during the time interval chosen (48 hours), expressed in function of the area of the primitive fragment of tissue. The ratio between the two relative growths,  $\frac{\text{Experiment}}{\text{Control}} = \frac{E}{C}$ , is also given. In Experiment 1 the tissues of both experiment and control were divided after the third passage. In Experiment 2 the tissues were divided in the same way after the first passage.

There is a close relation between the relative growths of the experiments and of the controls. This relation, expressed as ratios in the first experiment, was 0.93, 0.93, 0.89, 0.81, 0.83; and in the second,



0.85, 0.88, 0.70, 0.76, 0.82, 0.92. These figures express the fact that in the time elapsed between two passages, the amount of growth is very nearly the same both in the experiment and the control. A curve was plotted for both experiments in which these ratios were expressed in ordinates and the number of passages (48 hour intervals) in abscissæ.



TEXT-FIG. 2. Experiment 2. Ratio between the two relative growths, experiment and control.

#### DISCUSSION.

A heavy precipitate could be obtained from 10 cc. of diluted plasma by adding 1.2 to 1.6 cc. of a 1 per cent acetic acid solution. But the final product was too acid. 1 cc. of the acetic acid solution produced a precipitate which, after suspension in distilled water, had a hydrogen ion concentration between 6 and 6.3. The hydrogen ion concentration of serum was from 8 to 8.3 and that of embryo juice from 7.3 to 7.5. When the precipitate suspension was dissolved in serum and mixed with embryo juice, the hydrogen ion concentration of the mixture varied from 7.3 to 7.5.

The coagulation was brought about by the addition of embryo juice. The addition of calcium was not essential to promote coagulation. On the contrary, a precipitate formed and liquefaction of

the coagulum occurred after 24 hours. The presence of serum is generally necessary to prevent liquefaction of the coagulum. In the experiments in which fibrinogen suspension was mixed with Ringer's solution or embryo juice alone, without serum, coagulation took place. But progressive liquefaction began soon afterwards. When serum was added in small quantities to fibrinogen suspension and embryo juice, the stability of the clot could be maintained.

The suspension of fibrinogen was used in various concentrations. If the medium contained more fibrinogen than plasma, the tissues did not grow well. When the concentration of fibrinogen was decreased, the coagulum was not dense enough and liquefaction often occurred within 24 hours.

It was found that excellent growth took place in a medium composed of 12.5 per cent fibrinogen suspension, 37.5 per cent serum, and 50 per cent embryo juice. Coagulation occurred within 1 minute and the coagulum was still firm after 48 hours.

The character of the growth of the old strain of connective tissue in the medium was not different from that observed in plasma. The growth was slightly less extensive in the experimental medium than in the control. It was easy to extirpate the fragment of tissue from its medium after 48 hours, and to transplant it into another medium. After every passage the amount of new tissue was about as large in the experiment as in the control. There was no doubt that the culture could have been kept alive for several more generations. This shows the possibility of keeping a strain of connective tissue in a medium composed of serum, fibrinogen, and embryo juice in about the same condition as in plasma and tissue juice.

#### CONCLUSIONS.

A technique is described by which a medium composed of fibrinogen suspension, serum, and embryo juice may be made.

Fibroblasts grew in this medium about as well as in plasma and embryo juice.

A strain of connective tissue in this medium remained practically as active as the control for several passages.

# FLAGELLUM OF THE MICROORGANISM OF RAT-BITE FEVER.

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PLATES 81 AND 82.

(Received for publication, November 26, 1920.)

Since 1915 when Futaki and Ishiware found, independently, a microorganism as the cause of rat-bite fever, various investigators have published their results on this subject. The present paper is a report of observations recently made by the writer on the microorganism.

## *Material.*

Two strains were kept in the laboratory, one from an infected child, and the other from a rat. Besides these, four were obtained from fourteen rats. Each of these strains was injected cutaneously into two guinea pigs. In a few days the site of injection became hard, the lymphatic glands were swollen, and the animals developed fever and died. Death was caused by the injected organism.

## *Method.*

The blood of mice and guinea pigs injected with the strains described above was observed under dark-field illumination. The flagellum of the microorganism was fixed best with vapor of osmic acid (Figs. 1 to 4). Fixation with bichloride of mercury was unsuitable, since it caused contraction. Fixation with methyl alcohol was simple and brought out clearly a large flagellum. Before the cover-glass preparation was dried it was exposed to the vapor of osmic acid (1 gm. of osmic acid + 100 cc. of distilled water + 10 drops of 5 per cent bichloride of mercury) for from 30 seconds to 1 minute. Giemsa's solution or a modified reagent was used for staining. Gen-

erally the microorganism took a long time to stain, though the time required depended upon the power of the solution, which varied with the proportion of its components, "methylene azur," methylene blue, and eosin. The proportion of the components of the solution was not quite the same as that of Giemsa's solution, which did not give the best results, the commercial product often failing to stain the flagellum. A better solution for staining was obtained by adding a little alkali to Akashi's solution which is a modification of Giemsa's stain. It is worth mentioning that not only did the staining power of the preparation become very poor a few weeks after it was made, but also it was not always possible to obtain the same staining power, though the method of preparation was always the same. This point will be studied further.

#### OBSERVATIONS.

The microorganism consisted of the body and the flagella, as first recognized by Ishiwara and his coworkers and confirmed by many investigators. The body was short and thick; there were two or three windings which were thick, regular, and spiral, but not wave-like. Its length was about 3 microns. The microorganism moved energetically among the blood corpuscles when it was observed under the dark-field microscope, but its form was not changed during movement.

As to the number of the flagella, most writers have observed one flagellum at one end or at both ends. Matsusaki and his coworkers and Otawara observed one at one end and two at the other. In stained preparations the flagellum was observed at both ends of the microorganism or sometimes at one end only. In fresh preparations under dark-field illumination, however, they could always be seen at both ends. It is probable, therefore, that the flagella are generally present at both ends. In preparations fixed with methyl alcohol I found one flagellum at one or both ends. But in one preparation fixed with vapor of osmic acid, one flagellum was shown at one or both ends of some microorganisms, while many flagella were detected at one or both ends of others. These two types of microorganisms were always found by this method of staining, and the better the staining power of the solution the greater was the number with many

flagella. This was true even in the same material and preparation and seems to indicate that the number of flagella demonstrable depends on the staining power of the solution. These facts were confirmed by a study of the six strains already mentioned. The largest number of flagella counted was seven at one end. There were a few instances in which more than seven flagella were present, but they were so dense and tangled that they could not be counted. On the other hand, in one type of microorganism which showed one stained flagellum at one or both ends of the body, only one flagellum was found even when the staining power of the solution was best.

It therefore seemed advisable to investigate further the relation of these two types. 100 microorganisms with one flagellum at one or both ends and 100 with two or more flagella at one or both ends were studied. It was found that there was no relation between the length of the body or number of windings and number of flagella. It seemed possible, however, that the number of flagella might be related to stages of development. With this idea in mind streak preparations of blood taken from the ear vein of infected guinea pigs were made. Even in these cases two types were always found; that is, some microorganisms had one large flagellum at one or both ends and others had two or more flagella at one or both ends. But the ratio of the two types of microorganisms during one period showed no special relation to that at any other period.

The diameter of the end of the body of the microorganisms with one large flagellum gradually decreased to the flagellum, while in specimens with two or more slender flagella the ends of the body were blunt. Sometimes it was observed, however, that those with one slender flagellum were also blunt at the end of the body. In this case the number of flagella which were attached at the blunt end increased with better staining.

There were some peculiar specimens in which two flagella were fused into one (Fig. 8, *a* and *b*). In some instances the flagellum was divided into two or three parts at certain points (Fig. 8, *d* to *f*). Or again one flagellum was divided into two or three branches and one of the branches was again divided into two (Fig. 8, *f*). The part of the flagellum which was divided into two or three was generally

thicker than any other part. Furthermore, the diameters of the flagella of a microorganism might vary. In this case one flagellum which was larger than the others was sometimes divided into two or three (Figs. 5, *e*, 8, *d* and *f*, and 10, *a*).

Generally the flagellum was not straight; in the living specimen it was spiral. If the object-glass was skillfully moved in following a swift movement of the microorganism so as to keep it in the center of the dark-field, the movement became slow and the winding of the flagellum was readily observed, as the microorganism was weak to resist direct light; motion ceased even though the light was removed. In this case the flagellum became comparatively straight, L-shaped, or bow-like instead of spiral. Such was always true of stained preparations fixed with methyl alcohol. After fixation with osmic acid the flagellum was generally spiral just as in living microorganisms. Often the direction of the spiral was the same as that of the winding of the body. The spiral consisted of one and a half or two windings. When many flagella occurred at one end, the spiral of each was independent of the others. The direction of the spirals differed, but in many instances they faced one another. Two flagella, for example, which were extended at one end, were situated at an angle of  $180^\circ$  to each other (Fig. 5, *b* and *c*), or three flagella were kept at  $120^\circ$  (Figs. 5, *d* and 8, *e*).

The direction of the flagellum from the body varied with the movement. Futaki observed in streak preparations that the direction of the flagellum varied and thought that it was due to technique. This is not, however, always the case, because variation is observed even in a fresh preparation seen under the dark-field microscope. Often, however, the flagellum was extended in the same direction as the body as observed in a forward or backward movement (Figs. 1 and 5, *a*), though this did not always occur (Fig. 6, *a* to *c*). In some instances the flagellum was tangled around the body (Fig. 7, *b* and *c*), so that the microorganism appeared to have an undulating membrane. Furthermore, sometimes it was noticed that long microorganisms had one or two flagella in the center or near the center of the body. The center from which the flagellum originated was generally, though not always, small in comparison with other parts of the body. Moreover, this part was commonly slightly bent. In addition, two micro-

organisms, each of which had two or three windings, were connected with a thread and a flagellum at the connected ends (Fig. 10, *c* and *d*). Short microorganisms with one or more flagella at one or both ends were often observed (Fig. 9, *c* and *d*). These facts suggest that the microorganism divides transversely, though it is generally assumed that it divides longitudinally.

#### CONCLUSIONS.

The work of previous investigators was confirmed in that the microorganism of rat-bite fever was found to have flagella which are clearly visible by dark-field illumination and which can be stained. The best staining is obtained with alkalized Akashi solution, which is a modification of Giemsa's solution; the vapor of osmic acid gives the best fixation.

The number of demonstrable flagella seems to vary with the technique. Sometimes many slender flagella unite into one large one or one large flagellum divides into several smaller ones. It may be concluded that commonly many slender flagella occur at the ends and that these may unite into one or several large flagella. In the living microorganism the flagella appear to be spiral. Their form in fixed preparations depends upon the method employed. Some long forms have flagella arising at the middle of the body; this seems to indicate that division is transverse and not longitudinal as generally believed. The rigid body, the signs of transverse division, and multiple flagella seem to distinguish the forms reported here from spirochetes and indicate that they are spirilla.

The work was done under the direction of Professors Miyairi and Ogawa.

#### EXPLANATION OF PLATES

##### PLATE 81.

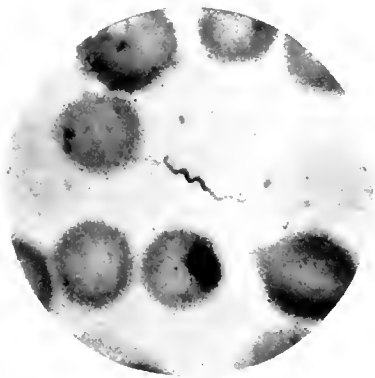
FIGS. 1 to 4. Photographs of microorganisms which were found in the blood of mice previously injected. Vapor of osmic acid fixation.  $\times 2,000$ .

##### PLATE 82.

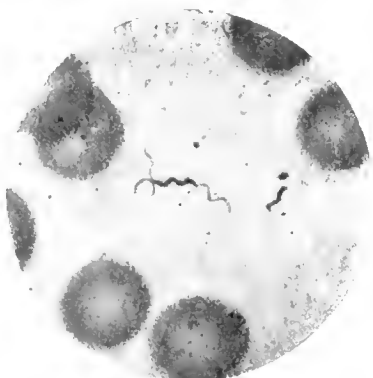
FIGS. 5 to 10. Free-hand drawings of microorganisms found in the blood of mice and guinea pigs previously injected. All flagella are drawn on the same plane, though they were not so in reality.



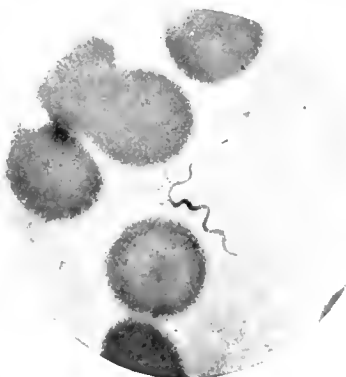




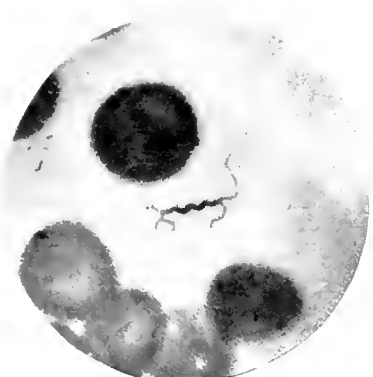
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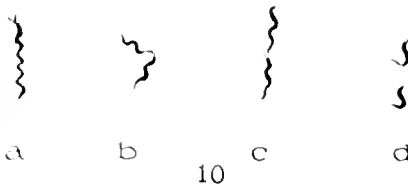
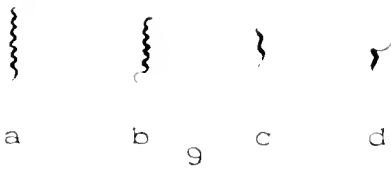
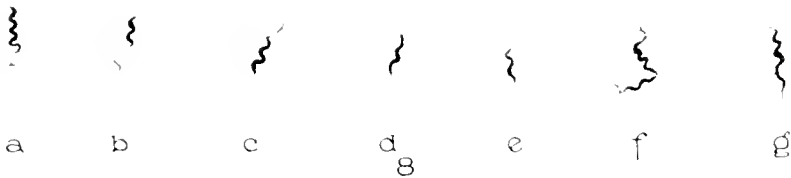
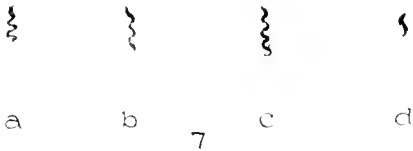
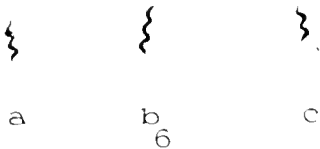
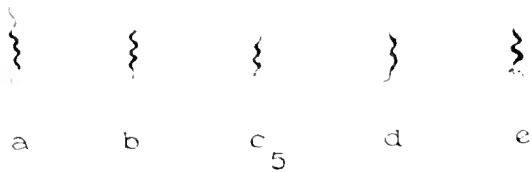
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(Adachi: Microorganism of rat-bite fever.)





(Adachi: Microorganism of rat bite fever.)



# IMMUNOLOGIC STUDY OF STRAINS OF BACILLUS PFEIFFERI ISOLATED FROM A CASE OF MENINGITIS.

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(Received for publication, February 3, 1921.)

The discovery of *Bacillus pfeifferi* by Pfeiffer in 1892, although made after the pandemic of 1899-90 had spent its force, was followed by rather general acceptance of the etiological relation of the organism to the disease. In the two succeeding decades it was found to have a widespread distribution in the upper air passages of normal individuals. In the next decade there were a number of minor and mild epidemics, in several larger American cities, of a disease clinically like influenza in which, not the influenza bacillus, but the streptococcus appeared to be the causal agent. These epidemics, however, were not associated with the dangerous complications nor did they have the high mortality of true epidemic influenza. With the passage of time since the 1899-90 pandemic the position of *Bacillus pfeifferi* as a pathogenic microorganism had become one of minor importance when the last pandemic made its appearance in 1918. The large amount of bacteriologic work done throughout the world in this epidemic still leaves the part played by *Bacillus pfeifferi* as the causative agent of pandemic influenza in doubt. Immunologic investigations have not helped to clear up this doubt.

That *Bacillus pfeifferi* may have well marked invasive and pathogenic properties is established by its occurrence as the only microorganism in a small proportion of cases of secondary bronchopneumonia and especially by its relation to sporadic cases of acute purulent meningitis.

Kuskow,<sup>1</sup> in 1895, estimated suppurative meningitis in the 1899-90 epidemic at 2.5 to 5 per cent of the fatal cases. That the reported cases from which he de-

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<sup>1</sup> Kuskow, N., *Virchows Arch. path. Anat.*, 1895, cxxxix, 406.

rived these figures were due to *B. pfeifferi* was not established by bacteriologic examination, and his figures cannot be accepted as giving the true incidence of meningitis in influenza. In 1904 Jundell<sup>2</sup> reported two cases and found in the literature twelve cases from which pure cultures of the bacillus were obtained. These cases dated from 1895. In those previous to this date the organism was not cultivated or the cultures were impure. In 1911 Wollstein<sup>3</sup> reported 49 cases, eight being cases studied by herself. There was a further group of nine reported cases in which the cultures were contaminated, and a group of eight which she considered doubtful. Torrey<sup>4</sup> reported two cases in 1916, making a total of 82 certain cases recorded up to that time; of these cases eight recovered. Lacy<sup>5</sup> in 1918 reported one case in which the organism was cultivated from the meninges at autopsy but not from the spinal fluid during life, and another case in which *B. pfeifferi* was grown in pure culture from the spinal fluid. Additional instances are reported by Nyberg,<sup>6</sup> Brown,<sup>7</sup> and Moody,<sup>8</sup> each of whom reported two cases; and by Aaser,<sup>9</sup> Packard,<sup>10</sup> Bhat,<sup>11</sup> and Hills,<sup>12</sup> each of whom reported one case.

The important point to be noted in connection with these 94 cases is that they occurred during the period between the two pandemics of 1899-90 and 1918-19. The small number of cases reported since the last epidemic began and experience in civil and military hospitals during the epidemic indicate that meningitis due to *Bacillus pfeifferi* is a sporadic infection of not very great frequency. While there seems to have been some increase in meningitis due to *Bacillus pfeifferi* since the height of the epidemic, in view of the widespread distribution of this organism the increase would appear not so great as might be expected if *Bacillus pfeifferi* were the cause of epidemic clinical influenza. Perhaps this fact may have some bearing upon any attempt to determine the part played by *Bacillus pfeifferi* in epidemic clinical influenza. Other points of interest which appear from the

<sup>2</sup> Jundell, I., *Jahrb. Kinderheilk.*, 1904, lix, 777.

<sup>3</sup> Wollstein, M., *Am. J. Dis. Child.*, 1911, i, 42.

<sup>4</sup> Torrey, R. G., *Am. J. Med. Sc.*, 1916, clii, 403.

<sup>5</sup> Lacy, G. R., *J. Lab. and Clin. Med.*, 1918-19, iv, 55.

<sup>6</sup> Nyberg, C., *Finska läk.-sällsk. handl.*, 1915, lvii, 1369.

<sup>7</sup> Brown, A., *Canad. Med. Assn. J.*, 1915, v, 1076.

<sup>8</sup> Moody, E. E., *J. Missouri Med. Assn.*, 1916, xiii, 328.

<sup>9</sup> Aaser, E., *Tidsskr. norske Lægefor.*, 1916, xxxvi, 393.

<sup>10</sup> Packard, F. R., *Ann. Otol., Rhinol. and Laryngol.*, 1916, xxv, 706.

<sup>11</sup> Bhat, K. S., *Lancet*, 1917, ii, 384.

<sup>12</sup> Hills, R., *New York Med. J.*, 1918, cvii, 345.

reported cases are the low age incidence, most of the cases occurring in very young infants or in children and only four of 94 in adults, and the high mortality, only nine of 94 cases having recovered.

#### OBSERVATIONS.

The isolation of five strains of *Bacillus pfeifferi* from a case of meningitis admitted to the Sarah Morris Hospital for Children gave an opportunity for studying the immunologic reactions of the micro-organism as obtained from a single case and for comparing definitely invasive strains with others, perhaps saprophytic, from the upper air passages. The results, together with the recently reported immunologic studies of others, appear to be of fundamental importance for an understanding of the biology of the organism and may have some bearing upon the possibility of developing a therapeutic antiserum.

#### *Isolation of Strains.*

The patient, a white, male infant age 17 months, on admission showed vomiting, twitching all over the body, fever, rigidity of the body, discharging right ear, and strabismus. The illness had begun with fever and vomiting 4 days before admission; muscular twitching appeared 2 days and rigidity 1 day before admission; strabismus developed on the day of admission. Death occurred on the 7th day in the hospital. The first culture of *Bacillus pfeifferi* was obtained from spinal fluid removed on the day of admission. This fluid had a cell count of 3,500 and all the characteristics of a fluid from acute purulent meningitis. On the following day cultures were made from the nose, throat, nasopharynx, ear, and blood.

A direct film of the spinal fluid stained by Gram's method showed a Gram-negative pleomorphic organism. There was variation in both size and morphology from coccus-like forms to rather long, slender bacilli. Direct films were not received in the laboratory from the nose, throat, nasopharynx, or ear.

Dewdrop colonies with the typical appearance of *Bacillus pfeifferi* appeared on blood agar cultures from the nose, throat, nasopharynx, blood, and spinal fluid, but not from the ear. Films made from

these colonies appeared, in the case of the spinal fluid culture, as Gram-negative pleomorphic organisms resembling those seen in the direct film. Films made from colonies of the other cultures showed typical, very small, Gram-negative bacilli fairly uniform in size. Subcultures were made on plain agar, Avery's<sup>13</sup> dextrose blood broth, and brown agar. No growth was obtained from the plain agar subcultures. In the Avery broth there was a rather slight growth showing some pleomorphism. In films from the spinal fluid colonies on the brown agar plates, the morphology was more typical than on whole blood agar. Colonies from the other sources tended to become more pleomorphic on brown agar than on whole blood agar. The brown agar was made by adding 2 per cent of defibrinated human blood to plain agar and heating to 80°C. long enough to produce a brown color. The growth on this medium was luxuriant, becoming more so after cultivation, which was in decided contrast to the very fine growth on blood agar. The ear cultures gave only a pure growth of *Staphylococcus albus*. From spinal fluid removed on the day after admission, when the cell count had increased to 10,000, *Bacillus pfeifferi* was again obtained in pure culture. Subcultures from selected colonies of the first spinal fluid culture and of the nose, throat, nasopharyngeal, and blood cultures on brown blood agar and on unheated whole blood agar were used for study.

#### *Pathogenicity.*

Determination of the pathogenicity of the strains was outside the scope of the investigation. The effect of an early generation of the spinal fluid culture was tried on a rabbit and a guinea pig, and some additional information upon the relative pathogenicity of the recently isolated strains from the other sources was obtained from the immunization of rabbits. A rabbit, two-thirds grown, received an intravenous injection of a salt solution suspension of the entire growth of a 24 hour brown blood agar culture of the second generation; the inoculation was without effect. Instillation of a heavy suspension of the spinal fluid strain into the nose of a guinea pig was also without effect. Since the invasive spinal fluid strain had proved to be

<sup>13</sup> Avery, O. T., *J. Am. Med. Assn.*, 1918, lxx, 17.



non-pathogenic for the first rabbit used, suspensions of living cultures of the other strains were used for the immunization of rabbits. The rabbits injected with the strains isolated from the blood, throat, and nasopharynx died after one injection. In all three instances, *Bacillus pfeifferi* of typical morphology and cultural characteristics was isolated from the heart's blood.

#### *Immunization.*

For the production of immune serums, rabbits one-half to two-thirds grown were used. The two animals which had withstood the effect of the first intravenous injection of living suspensions of the spinal fluid and nose strains were subjected to further injections. Since the strains isolated from the blood, throat, and nasopharynx had proved fatal to rabbits injected intravenously with suspensions of living organisms, the immunization of other rabbits was begun with suspensions of these strains killed by heating to 80°C., living suspensions being used later. An additional rabbit was immunized with the spinal fluid strain. The intravenous injections were repeated every other day. When trial bleedings from the ear vein gave satisfactory agglutination, the animals were bled from the heart<sup>14</sup> and the serums were separated. In this way there were prepared two serums against the spinal fluid organism, and one serum against each of the other strains. These serums were used for a study of agglutination, complement fixation, and phagocytic activity.

#### *Agglutination.*

The macroscopic method was used. Each immune serum was used with its homologous strain and with the four heterologous strains. The serum was used in dilutions of 1:4, 1:8, 1:16, and so on by geometrical progression to 1:4,096. To each dilution of serum an equal quantity of *Bacillus pfeifferi* suspension was added, the total volume of fluid being 0.5 cc. This suspension was obtained by washing down twenty-four cultures with normal salt solution. The growths, since they were quite cohesive, were well shaken and then centrifuged for a few minutes to throw down the larger particles, and the supernatant fluid was used. A test was incubated at 56°C. for

<sup>14</sup> This was always done under anesthesia.

4 hours and placed in the ice box over night; another was incubated at 53°C. for 20 hours. Neither of these, however, proved so satisfactory as material incubated at 37°C. for 2 hours and placed in the ice box over night. Since it was feared that the strains might die out during subsequent work, cultures were made on brown blood agar slants in Blake flasks. From the luxuriant growths thus obtained large amounts of suspension were made, the organisms being killed by heating to 80°C. It was hoped that the use of such stock suspensions throughout all the work might give more satisfactory results. It was soon found, however, that agglutinations with these older suspensions were unsatisfactory. A slightly turbid fresh suspension of a 24 hour living culture gave better results than a more turbid suspension. The results of the agglutination tests are given in Table I,

TABLE I.  
*Agglutination.*

Suspensions of <i>B. Pfeifferi.</i>	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso- pharynx.	
Spinal fluid.....	1:4,096	1:1,024	1:64	1:64	1:64	1:16	0
Blood.....	1:8	1:32	1:128	1:4	1:4	1:4	0
Nose.....	1:64	1:128	1:32	1:64	1:32	1:8	0
Throat.....	0	0	0	0	1:1,024	0	0
Nasopharynx.....	1:64	1:64	1:16	1:256	1:8	1:512	0

the figures being the highest dilution of serum at which clumping was apparent to the naked eye.

The results of the agglutination experiments show a wide variation in the titer of the serums for their homologous strains. Whether this is due to differences in agglutinability of the strains, to variations in agglutigen content of the strains, or to variations in the response of the animals used it is impossible to decide. With the exception of the serum against the strain from the nose each serum agglutinates best its homologous strain. Each serum except that against the throat strain gives also cross-agglutination with the heterologous strains in low dilutions of serum. The serums against the spinal fluid organism have the highest titer, that against the strain from the throat is most specific, whereas the serums against the strains from

the nose and nasopharynx are least specific. It was originally intended to perform absorption agglutination experiments, but the exhaustion of the supply of serums and the later loss of some of the strains prevented this.

### *Complement Fixation.*

In the complement fixation experiments two series of tests were set up. In the first, each immune serum was used with the spinal fluid strain. This exhausted the supply of serums, so that cross-fixation tests with the remaining strains as antigens could not be done. There was left just enough immune serum from the rabbits injected with the nose and throat strains to set up these serums with their homologous strains. After the original supply of serums was exhausted, an attempt was made to obtain a fresh supply by reinmunizing the original animals with stock killed suspensions of the strains which had been used before. This second lot of serums was not so satisfactory as the first but was used in a second series of experiments in which each serum was set up with all the strains. Because of the loss of several of the strains it was necessary to use as antigens the stock killed suspensions which had been prepared at the beginning of the work.

Undiluted serums heated at 56°C. for 30 minutes were used. In the first series the antigens were made of unheated bacterial suspensions such as were described under Agglutination. The system used was the anti-sheep rabbit in one-tenth the volume of the original Wassermann test. The serum, antigen, and complement, which consisted of two units of fresh guinea pig serum, were incubated at 37°C. for 1 hour. Two units of previously titrated anti-sheep amboceptor and a 5 per cent suspension of sheep corpuscles were then added and the whole was incubated for 1 hour. Antigen, serum, and hemolytic control tests were made each time. Serum and complement were kept constant; the antigens were varied, the figures given in Tables II and III being the fractions of the anticomplementary unit which gave complete inhibition of hemolysis.

As has been found to be the case in comparative studies of agglutination and complement fixation with other species of bacteria, the complement fixation experiments with *Bacillus pfeifferi* show a lesser

degree of specificity than do the agglutination reactions. An unexpected result is the degree of fixation which normal rabbit serum gave with the different antigens. The serum of a number of different normal rabbits was used, but always with the same result. In the second series, in which the older stock suspensions were used as antigens, this phenomenon was even more marked. It would appear to be due to a property of the antigen rather than of the normal serum;

TABLE II.  
*Complement Fixation.*

Antigens of <i>B. pfeifferi</i> .	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso- pharynx.	
Spinal fluid.....	$\frac{1}{256}$	$\frac{1}{256}$	$\frac{1}{4}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{64}$	$\frac{1}{4}$
Nose.....				$\frac{1}{64}$			$\frac{1}{4}$
Throat.....					$\frac{1}{64}$		$\frac{1}{4}$

TABLE III.  
*Complement Fixation.*

Antigens of <i>B. pfeifferi</i> .	Immune serums.						Normal rabbit serum.
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso- pharynx.	
Spinal fluid.....	$\frac{1}{32}$	$\frac{1}{128}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{8}$
Blood.....	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$	$\frac{1}{8}$
Nose.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{64}$	$\frac{1}{64}$	$\frac{1}{16}$	$\frac{1}{8}$
Throat.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{8}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{8}$
Nasopharynx.....	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{32}$	$\frac{1}{16}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{16}$

the antigen appeared to be somewhat anticomplementary when used with normal rabbit serum, although it had no such action when added to the hemolytic system in the absence of rabbit serum.

### *Opsonins.*

The opsonic index of each immune serum with its homologous influenzal strain was determined. Equal parts of suspensions of living *Bacillus pfeifferi*, normal rabbit leucocytes (collected in 0.2 per cent sodium citrate, washed, and suspended in normal saline

solution), and inactivated serum were mixed and incubated at 37°C. for 30 minutes. 50 polymorphonuclear leucocytes were counted and the number of cells taking part in phagocytosis was noted and compared with a similar series in which normal rabbit serum replaced the immune serum. The strain isolated from the blood was also set up with all the immune serums. The results of these experiments are given in Table IV. Because of the low opsonic indexes obtained in these experiments further work in this direction was not done.

With the exception of the first spinal fluid serum, all the serums used were of the second lot. The opsonin experiments show much the same kind and degree of variation as the agglutination tests. The spinal fluid serums, which had the highest agglutinin titers, have

TABLE IV.

*Opsonins.*

Suspensions of <i>B. Pfeifferi</i> .	Immune serums.					
	Spinal Fluid 1.	Spinal Fluid 2.	Blood.	Nose.	Throat.	Naso- pharynx.
Spinal fluid.....	2.1	1.9				
Blood.....			1.4			
Nose.....				1.8		
Throat.....					1.5	
Nasopharynx.....						1.6
Blood.....	1.3	1.2	1.4	1.1	1.0	1.0

also the greatest opsonic activity. In the series in which the different serums were used with the same bacterial suspension, the organism being the strain from the blood, the action of the serum is best with the homologous organism, but the differences are not very marked.

## DISCUSSION.

Immunologic studies of *B. Pfeifferi* have led to conflicting results. Park,<sup>15</sup> by agglutination methods, found that cultures from different cases, even in the same locality, differed essentially or completely from each other. Isolations from the same case were usually identical, but different strains could occasionally be obtained from the same case. The differences noted were stable and persisted after

<sup>15</sup> Park, W. H., *J. Am. Med. Assn.*, 1919, lxxiii, 318.

subculturing. Huntoon and Hannum<sup>16</sup> came to exactly opposite conclusions and claimed an intimate relation for different strains, even when these were isolated in widely separated regions. Their work was based upon a small number of strains. Their agglutinin titers with three monovalent serums and four strains were low, the maximum of 1:640 being reached only once. The absorption experiments gave a decrease in titer varying from 1:10 to 1:80. Small and Dickson<sup>17</sup> also found strain relations by agglutination methods and arranged their strains into four groups. The number of strains used by them was, however, only ten, of which three fell into one group, four into another, while the two remaining groups were each represented by only one strain; there was one additional strain which could not be placed in any of these groups because of its inagglutinability. The propriety of setting up groups with only single representatives must appear doubtful; we believe that the study of a larger number of strains might have increased the number of single strain groups indefinitely. Valentine and Cooper,<sup>18</sup> in agglutination and absorption experiments on 171 strains from autopsies and from the upper air passages of patients with influenza, found little or no evidence of identity and concluded that under *B. Pfeifferi* is included a heterogeneous group of organisms, among which there may be small subgroups. Six strains isolated from as many members of a single family, all taken ill with influenza at about the same time, were all different. Uthelm<sup>19</sup> isolated thirty strains from thirty patients and tested the agglutination of the patients' serums with the homologous strains. Eleven (36 per cent) gave a positive agglutination in dilutions which varied from 1:20 to 1:160. In cross-agglutination experiments only one strain was agglutinated by heterologous serum, giving a positive result with the serums of three patients in 1:40 dilution. Uthelm concluded that "each strain seemed to be individual in its immunologic reaction." Bell<sup>20</sup> studied the agglutination reactions of thirty-six strains, isolated by pharyngeal culture, with twenty-seven monovalent serums. His results are particularly valuable because of the high titers which the serums developed. Cross-agglutination occurred frequently in low dilution, but in only a few instances in higher dilutions. Absorption experiments also showed such variation that grouping of strains was impossible; the organisms used for absorption might absorb all, part, or none of the agglutinin for other strains. Strains isolated from two brothers were identical; from one individual two strains were isolated which were identical with two isolated from another individual. Three strains isolated from a single individual were quite distinct from one another. Although identical strains may occur, Bell concluded that "the influenza bacillus represents a heterogeneous group of

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<sup>16</sup> Huntoon, F. M., and Hannum, S., *J. Immunol.*, 1919, iv, 167.

<sup>17</sup> Small, J. C., and Dickson, G. K., *J. Infect. Dis.*, 1920, xxvi, 230.

<sup>18</sup> Valentine, E., and Cooper, G. M., *J. Immunol.*, 1919, iv, 359.

<sup>19</sup> Uthelm, K., *J. Infect. Dis.*, 1920, xxvii, 460.

<sup>20</sup> Bell, H. H., *J. Infect. Dis.*, 1920, xxvii, 464.

organisms." Cooke<sup>21</sup> was likewise unable to find any antigenic relation when sixteen of the strains isolated by Bell were used as antigens in the complement fixation reaction with patients' serums.

The study of five strains isolated from the same case, of which strains two were definitely invasive, appeared to offer better possibilities of obtaining knowledge of the biology of *Bacillus pfeifferi* than the study of many strains from a large number of cases. Whether the primary invasion in the present case was one of the blood stream or of the meninges, it is impossible to say. We believe it more probable that the meninges were invaded first and directly from the upper air passages, and that the blood was invaded from the meninges. The five strains isolated show distinct immunologic differences. The strains isolated from the nose and the nasopharynx show a greater apparent relation than any other two strains, and there may be some slight relation between the spinal fluid and the nose strains. The relation in each instance, however, is less marked than that which occurs among certain members of the colon-typhoid intermediates which are considered to be distinct species. Unfortunately, absorption experiments could not be done, but it is believed that they would not have added much of importance since cross-agglutination was so slight. The opsonin studies, although phagocytosis was not strikingly increased by the immune serums, show much the same differences as those obtained by agglutination. Since the results given were obtained by comparing the action of immune serum on normal leucocytes with that of normal serum, the phenomenon of spontaneous phagocytosis in the absence of serum, noted by Davis,<sup>22</sup> may be excluded. The pathogenicity of the five strains for rabbits also varied, although no great importance may be attached to these results because of the small number of animals used. Suspensions of living cultures of the blood, throat, and nasopharynx strains caused death from bacteremia. The nose and the spinal fluid strains were non-pathogenic for rabbits in large doses.

Our results, as well as those of Park,<sup>15</sup> Valentine and Cooper,<sup>18</sup> Uthelm,<sup>19</sup> Bell,<sup>20</sup> and Cooke,<sup>21</sup> are directly opposed to those of Hun-

<sup>21</sup> Cooke, J. V., *J. Infect. Dis.*, 1920, xxvii, 476.

<sup>22</sup> Davis, D. J., *J. Am. Med. Assn.*, 1907, xlviii, 1563.

toon and Hannum,<sup>16</sup> and of Small and Dickson.<sup>17</sup> The evidence obtained from strains isolated from the same case or from different cases against identity of strains, and even against any close immunologic relation of strains, appears overwhelming. What bearing these results have upon the question of the etiological relation of *Bacillus pfeifferi* to the epidemic disease, it is difficult to say. Park<sup>15</sup> maintains that epidemic strains must be biologically identical if they are to be considered to have any causal connection with the disease. For most species of bacteria this is undoubtedly true. With *Bacillus pfeifferi* the question arises whether it may be possible that the wide variations in strains indicate biological instability which, under proper conditions, becomes an important factor in the causation of epidemics. In spite of the fact that immunologic characters become fixed upon cultivation of *Bacillus pfeifferi* outside the body, it is difficult to exclude the possibility that the organism is so labile that it may undergo changes under natural conditions in the human body. Although different strains, even from the same case, may show wide variations in experimental immunologic reactions, we believe that such strains are not so distinct from each other as are the various members of the colon-typhoid intermediate group, for instance. While the present case yielded five strains which have little in common except cultural and morphologic characters, it is probable that the subculturing of more colonies from the original cultures would have yielded a still larger number of immunologically different strains. However great the differences between the strains which were isolated, it is difficult to believe that these strains are not genetically the same.

If, as appears to be the case with antimeningococcus serum, the agglutinin and opsonin titers of an immune serum are a measure of the therapeutic value of the serum, the multiplicity of strains or races of *Bacillus pfeifferi* would seem to render impossible the preparation of a serum which might be of value in influenzal meningitis. Such a serum was prepared by Wollstein<sup>23</sup> at The Rockefeller Institute and was found to have definite value in experimental influenzal meningitis of monkeys. The serum had a low agglutinin titer but

<sup>23</sup> Wollstein, M., *J. Exp. Med.*, 1911, xiv, 73.



markedly increased phagocytosis. The immunologic relation of the organisms used in the experimental infection to those used in the preparation of the immune serum is not apparent from her report. The use of this serum in one of the cases reported by Torrey<sup>4</sup> was followed by recovery. Packard's<sup>10</sup> case also received this serum and recovered. Hills'<sup>12</sup> patient who did not receive the serum until the 4th day of the disease died on this day. Although one hesitates to draw conclusions from such a small number of cases, recovery may have been due to the serum, since the usual mortality of influenzal meningitis is approximately 90 per cent. In view of later immunologic studies, there is no reason to suppose that the organisms from the recovered cases would have been found to be identical with the strains used in the preparation of the serum. The present opsonin experiments with monovalent serums indicate strain variations comparable with those obtained in the agglutination experiments. The opsonin titers of our serums were so low that we do not attach great importance to the results obtained; it is possible that serums with higher opsonin values might have shown less variation between strains and a greater degree of group reaction. It is also possible, if strains of *Bacillus pfeifferi* are genetically related but immunologically distinct, that the therapeutic value of a serum in meningitis may depend upon properties other than those which can be measured by agglutination or opsonin reactions.

#### SUMMARY.

Five strains of *Bacillus pfeifferi* were isolated from a case of meningitis. These strains came from the spinal fluid, blood, nose, throat, and nasopharynx.

Immunologic reactions show no definite relations between these strains, although those from the nose, throat, and nasopharynx might be presumed to be related to one another. It is also presumable that the spinal fluid strain was derived from the upper air passages, and that the blood was invaded from the meninges. In spite of immunologic differences, it is believed that the five strains were genetically related.

The variations in these five strains from a single case are as great as those which have been found by others for strains from different cases, or individuals.

As determined by immunologic reactions, the number of so called strains of *Bacillus pfeifferi* is apparently limited only by the number of cultures which have been or might be isolated. It is inconceivable that under the designation *Bacillus pfeifferi* is included a heterogeneous mixture of innumerable distinct races of bacteria; there must be some biological relation which cannot be established by agglutination, complement fixation, or opsonin reactions.

The variations which have been noted may be an indication of a degree of instability which may have some bearing upon questions relating to the epidemiology of *Bacillus pfeifferi* infections and to the serum therapy of influenzal meningitis.

The strain variations which have been shown to exist by immunologic methods do not support the theory of the etiological relation of *Bacillus pfeifferi* to epidemic influenza, unless it can be shown that such variations are due to instability of the organism. The fact that the incidence of meningeal involvement was only slightly increased during the last pandemic is also evidence against the causal relation of the organism to the pandemic disease.

# THE INCIDENCE OF BLACKHEAD AND OCCURRENCE OF HETERAKIS PAPILLOSA IN A FLOCK OF ARTI- FICIALLY REARED TURKEYS.

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(Received for publication, February 16, 1921.)

During the season of 1920 a flock of turkeys reared in incubator and brooder, and on new soil, has provided further data on the incidence of blackhead under such circumstances and has furnished additional evidence that this method is a successful means of rearing turkeys. Interesting data have also been obtained on the occurrence of the worm (*Heterakis papillosa*) shown in previous work to be a factor in the production of this disease and some evidence has been obtained on the source of this worm in the environment under which the experiments were conducted.

The ground on which the flock was reared was in a large, well fenced horse paddock which had not been used for turkeys or chickens for a period of more than 3 years. The soil was plowed and sown in the spring. The old turkeys and poultry were confined in enclosures at a distance from the paddock and precautions were taken to prevent attendants carrying infection from these to the flock in the paddock. As in the previous field experiments no attempt was made to exclude wild birds.

Three lots of eggs were incubated in commercial incubators. These yielded 85 poults. The lots were brooded separately and kept apart in coops and enclosures of their own until August 9 when they were combined into one flock and given the run of the paddock, within which the enclosures used up to this time had been located. Lot 1 was hatched May 30, Lot 2 June 9, and Lot 3 June 22. They were placed outdoors June 9 and 15, and July 1 respectively.

Up to October 1, nine had died of miscellaneous causes and four from blackhead. Twenty-eight in all had been removed at intervals

for experimental purposes. The remaining birds were in a flourishing condition. Of those that died of miscellaneous causes, one was trampled to death, in two the cause of death remained undetermined, two died of general weakness, two of leg weakness, one of typhlitis, and one of enteritis. After October 1, one other case of blackhead occurred and one more bird was removed for experimental purposes.

It will be noted that, in all, five cases of blackhead occurred in the flock. The dates on which the birds became sick were July 14, September 3, 15, 17, and October 5. The contents of one cecum in the first case were washed and sedimented for worms and one young *Heterakis papillosa* was found. The second case was not examined for worms. The contents of both ceca of the three remaining cases were washed and sedimented, and ten *Heterakis* small to full grown were found in one, one young and one full grown in another, and two full grown specimens in the third. Three of the birds were examined for coccidia with negative results.

With the exception of four birds retained for breeding, the remainder of the flock consisting of 42 birds was killed for food purposes during November and December. Data on these turkeys are given in Table I. The organs of all were examined for lesions of blackhead and for scars. The examination proved entirely negative. None of these birds had at any time shown symptoms of illness. All were normal at autopsy with one exception, in which the mucosa at the tips of the ceca was congested and pigmented.

The degree of infestation with *Heterakis* was determined by washing and sedimenting the cecal contents. It will be noted from the table that five were free of worms. Eleven turkeys harbored one worm each; eight, two worms; three, three worms; six, four worms; one, seven worms; three, eleven worms; and one, fourteen worms. As evidence of the difficulty the parasite has in establishing itself in a flock on virgin soil, it is of interest to note the number of cases in which either males alone or females alone were present. In twelve birds only males were present and in six only females. Further obstacles are presented to the multiplication of the parasite in that males alone presumably might occur in one cecum and females in the other, and moreover when both sexes occur together, individuals of both in the same stage of development might not be present, a circumstance not favorable to reproduction.

TABLE I.  
*Data on Turkeys Killed for Food.*

No. of turkey.	Date killed.	Live weight.	Color.* Sex.*	No. of <i>H. papillosa</i> present.
	1920	lbs.		
430	Nov. 4	9½	B.	1 grown, 1 almost grown.
431	" 20		" M.	2 " females.
432	" 20	13¾	" "	None.
433	" 20	9½	W.; F.	"
434	" 20	8¼	B.; "	"
435	" 22	13¾	W.; M.	2 adult males.
436	" 22	13	" "	2 " females, 1 male.
437	" 22	12	" "	4 " " 6 nearly to full grown, 1 young male.
438	" 22	8	" F.	1 adult female, 3 young to grown males.
439	" 23	16½	B.; M.	1 young male.
440	" 23	13½	" "	3 grown females, 4 adult males.
441	" 23	14	" "	3 " males, 1 young female.
442	Dec. 18	13	" "	1 male nearly grown.
443	" 18	13½	" "	5 males and 5 females nearly to full grown and a young stage 2-3 mm. long.
444	" 18	8¼	W.; F.	1 grown male.
445	" 18	8¼	B.; "	4 adult males.
446	" 18	9¼	" "	1 male and 1 female, adults.
447	" 20	15	W.; M.	1 grown male.
448	" 20	8¾	B.; F.	3 " females and 8 males nearly to full grown.
449	" 20	14¼	" M.	1 adult female.
450	" 20	14	" "	1 grown male.
451	" 20	16¼	W.; "	3 adult females and 1 male nearly grown.
452	" 21	9	B.; F.	2 grown females.
453	" 21	9¾	W.; "	1 " female, 1 adult male.
454	" 21	8	B.; "	14 males and females, nearly to full grown
455	" 21	9½	" "	2 grown males.
456	" 22	10½	" "	1 nearly grown female.
457	" 22	10½	" "	1 " " male.
458	" 22	8½	" "	3 females nearly to full grown.
459	" 22	8½	" "	3 adult females, 1 male nearly grown
460	" 22	10½	" "	1 grown female, 2 adult males.
461	" 23	12	W.; M.	1 " male.
462†	" 23	14¼	B.; "	None.
463	" 23	9¾	" F.	4 worms.
464	" 28	14	" M.	1 nearly grown male.
465	" 28	16	" "	2 " " males.
	" 28	16¼	" "	None.
	" 28	16½	" "	1 nearly grown female.

\* B. indicates bronze; W., white; M., male; F., female.

† Mucosa of tips of ceca greatly congested and pigmented.

As evidence that the flock of turkeys was not one specially resistant to blackhead it will be of interest to consider the history of a number withdrawn during June and July and exposed to an old flock. The data on the incidence of blackhead and the occurrence of *Heterakis* in these furnish a striking contrast to what was observed in those not exposed.

Of twenty-nine birds removed for experimental purposes, sixteen were penned with the old flock beginning in July. All became

TABLE II.  
*Data on Turkeys Exposed to Old Flock.*

No. of turkey.	Date exposed.	Date sick.	Blackhead.	Cecal contents washed and sedimented.	No. of <i>H. papillosa</i> present.
	1920	1920			
400	July 12	Aug. 2	+	Both ceca.	59 young to grown.
401	" 12	" 16	+	" "	21 "
402	" 12	" 8	+	" "	7 " to grown.
403	" 12	July 26	+	" "	About 25 young.
404	" 12	Aug. 16	*		
405	" 12	July 29	+	Both ceca.	11 young.
406	" 12	Aug. 8	+	" "	30 "
407	" 12	" 2	+	" "	90 "
408	" 19	" 6	+	†	
409	" 19	" 8	+	Both ceca.	100 young.
410	" 19	" 23	+	†	
411	" 19	" 16	*		
412	" 12	July 28	+	Both ceca.	7 young.
413	" 12	Aug. 8	+	" "	7 nearly grown.
414	" 12	" 23	+	" "	49 young to grown.
415	" 12	" 16	+	One cecum.	Many, mostly young.

\* Survived.

† No examination for worms.

sick within 42 days (Table II). Two survived and there is every reason to believe that they underwent an attack of blackhead, since both were sick for about 7 days. The diagnosis of blackhead in the others was confirmed at autopsy. In washing and sedimenting the contents of the ceca the number of *Heterakis* present was determined. In eight birds the number of worms ranged from 21 to 100. In the four remaining ones examined, the number found was 7 to 11. It should be stated that the method used in collecting the worms

would not lead to the detection of very young stages. Ten of the birds were examined at autopsy for coccidia with negative results.

Since the flock of normal turkeys was protected from infestation with *Heterakis papillosa* from other turkeys and chickens, the source of this parasite becomes a question for consideration. We have been fortunate in locating at least one of the sources of this infection in the ring-necked pheasant, present in this locality. In the previous fall a pheasant killed on a neighboring farm harbored specimens of this worm. Pheasants have been observed from time to time on the Institute farm and on a number of occasions they have been seen in close proximity to the laboratory and in the paddock in which this year's flock was reared. In June of this season attendants reported on a number of occasions a pair of pheasants present in this paddock. On November 18, a pheasant that had fallen a victim to hunters was found dead in the paddock and at autopsy specimens of *Heterakis papillosa* were found in the ceca. Considering the resistant character of the ova of this parasite, there seems little question that this bird, although present in small numbers, is capable of maintaining a certain degree of soil infestation with this parasite. Fortunately, this does not appear to be sufficiently concentrated to interfere with the successful rearing of turkeys when the soil has been subjected to ploughing and cultivation incident to planting. It will, however, be a safe practice to discourage the visits of this bird.

*Experiment on the Persistence of the Ova of Heterakis in the Soil.*

An enclosure that had proved infectious to turkeys during the season of 1919<sup>1</sup> remained occupied by recovered cases until January 21, 1920, at which time it became vacant and remained closed until June 28, a period of a little over 5 months. This covered a time of exceptionally severe winter weather with much ice and snow. On the latter date, four healthy turkeys, 29 days old, from the normal flock referred to above were placed in the enclosure. These turkeys contracted blackhead in 11, 21, 23, and 28 days respectively. *Heterakis* was present in all. The contents of both ceca were washed and

<sup>1</sup> Smith, T., and Graybill, H. W., *J. Exp. Med.*, 1920, xxxi, 633, Experiment 12, b.

sedimented in the case of three of the birds. In one, seven worms were collected, and in the remaining two, many were found. Only one cecum of the fourth bird was examined and twenty-seven young *Heterakis* were collected.

Although the enclosure during the time it was unoccupied remained accessible to wild birds on the wing, the conditions for visits of hosts of *Heterakis* were on the whole unfavorable. Circumstantial evidence that the ova in the soil had survived and had not been introduced during the interval is furnished by the fact that the number of worms found per bird corresponded with that of the poults penned with the old flock and not with that of the flock on new ground, which was much more favorably located for the visits of birds.

#### SUMMARY.

In a flock of artificially reared turkeys originally consisting of 85 birds and reduced during the summer and fall by deaths and withdrawals for experimental purposes to 42 birds, five cases of blackhead occurred. These appeared during the months of July, September, and October. In four, *Heterakis* was searched for and found. In 38 birds from this flock killed for food during November and December, five harbored no *Heterakis*, and the rest carried light infestations.

Of sixteen healthy birds withdrawn from the above flock during July and placed with a flock of older birds which had passed through this disease in former seasons, all contracted blackhead and fourteen died of the disease. The infestation with *Heterakis* was, as a rule, high, reaching a hundred specimens in some cases. In general, it appears that a high infestation with *Heterakis* is correlated with a high incidence of blackhead, a relation that had already been inferred in feeding experiments. In both of these groups no other species of worm was found in the ceca, and in instances in which examinations for coccidia were made none was found.

Pheasants have been incriminated as a source of infestation with *Heterakis papillosa* in artificially reared flocks.

In an artificially reared flock 38 birds that had never been ill, when killed in November and December, failed to show lesions of blackhead or evidence in the nature of scars that they had passed through an attack of the disease.



Infectious soil that had remained unoccupied by turkeys and chickens for a period of 5 months beginning in the depth of a severe winter still harbored viable ova of *Heterakis* and proved highly dangerous to young poults.

These experiments and observations fail to throw any light on the source of the protozoan parasite (*Amæba meleagridis*) which causes the fatal lesions of blackhead.



## A USEFUL HEART METHOD.

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PLATES 83 AND 84.

(Received for publication, February 21, 1921.)

In order to accomplish certain experiments upon the relation of the pulmonary circulation to the air space in the lungs, it became necessary to devise a method in which the heart was exposed and the breathing normal. The procedure described below fulfills these requirements.

Under urethane anesthesia (2 gm. per kilo) and artificial respiration, an elliptical section is cut from the anterior aspect of the thorax of a cat. This piece must be taken out neatly and should leave the uppermost and lowest rib attachments to the sternum and sternal cartilage intact. The heart is then seen beating within the pericardium and the removed section of chest wall should be but slightly larger than the heart itself, so that the organ is framed by the margin of the opening. The anterior aspect of the pericardium is now slit exactly in the midline, and the edges of the pericardial opening attached to the margin of the thoracic window by three strong sutures on either side and one at each extremity. Then, by use of a long continuous suture, the pericardium is sewed to the margins of the window and is thus made to close the opening completely. It will be found to do more than this; that is, there will be a considerable amount of loose pericardial membrane below the heart, a desirable situation since the suturing should not involve serious tension at any point.

The chest wall, which has been closed in this manner anteriorly by the fibrous pericardial membrane, is now stretched laterally by traction on the stay sutures first placed, and superiorly and inferiorly by the stay sutures at the ends of the incision. The retraction so accomplished throws the heart forward where it may be handled with

greater readiness, and takes up the pericardial slack so that a reasonably rigid anterior chest wall is provided. Fortunately, the vessels entering the heart do so posteriorly, and lifting the heart forward in the manner described does not seem to affect its filling. Before discontinuing artificial respiration it is advantageous to withdraw air from the thorax with a syringe in order to assist in the reestablishment of negative intrathoracic pressure.

Fig. 1 shows the preparation as it appears when finished. Obviously one may readily adjust a cardiometer in such a heart technique and manipulate the cardiac vessels, etc., in connection with procedures in which it may be desirable to measure normal respiration at the same time.

Fig. 2 is a record made during the course of an experiment in which a urethanized cat, with heart exposed in the manner described, remained in good physiological condition from 10.35 a.m. to 3.20 p.m., and was then killed with chloroform. The lungs were not edematous at death and the animal would apparently have done well through many more hours.

In this tracing it is noticeable that the blood pressure never reached so high a level after as before operation, and this has invariably been our experience in using the method described. The height attained, however, has always been as great as under artificial respiration, and frequently has exceeded that level. The technique has been employed in the cat alone, but its ease of accomplishment in this animal leaves little doubt of its usefulness in other laboratory animals.

#### EXPLANATION OF PLATES.

##### PLATE 83.

FIG. 1. Method of exposing the heart under normal breathing.

##### PLATE 84.

FIG. 2. Upper tracing, respiration; inspiration written downward; middle tracing, arterial blood pressure; lower tracing, signal magnet  $\frac{1}{2}$  minute intervals. Tracing I, normal blood pressure. Tracing II, transition from artificial respiration to normal respiration. Tracing III, condition at 11 a.m. Tracing IV, condition at 12.35 p.m.

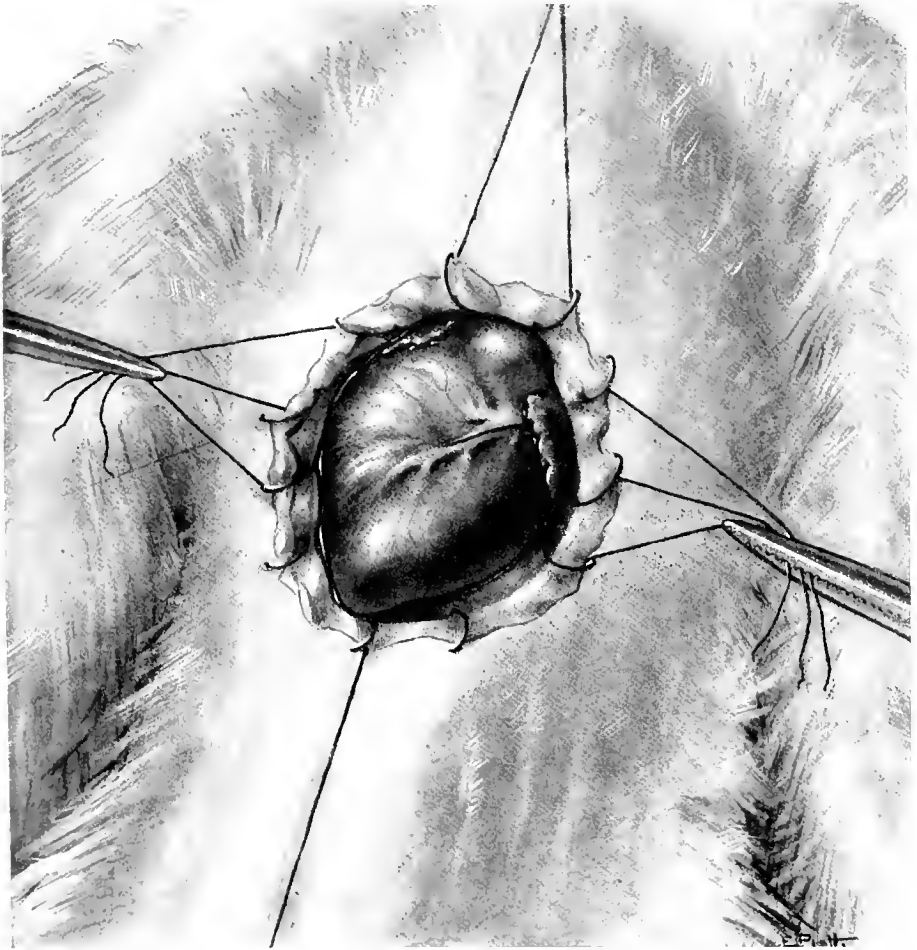


FIG. 1.

(Drinker; Useful heart method.)



676<sup>13</sup>

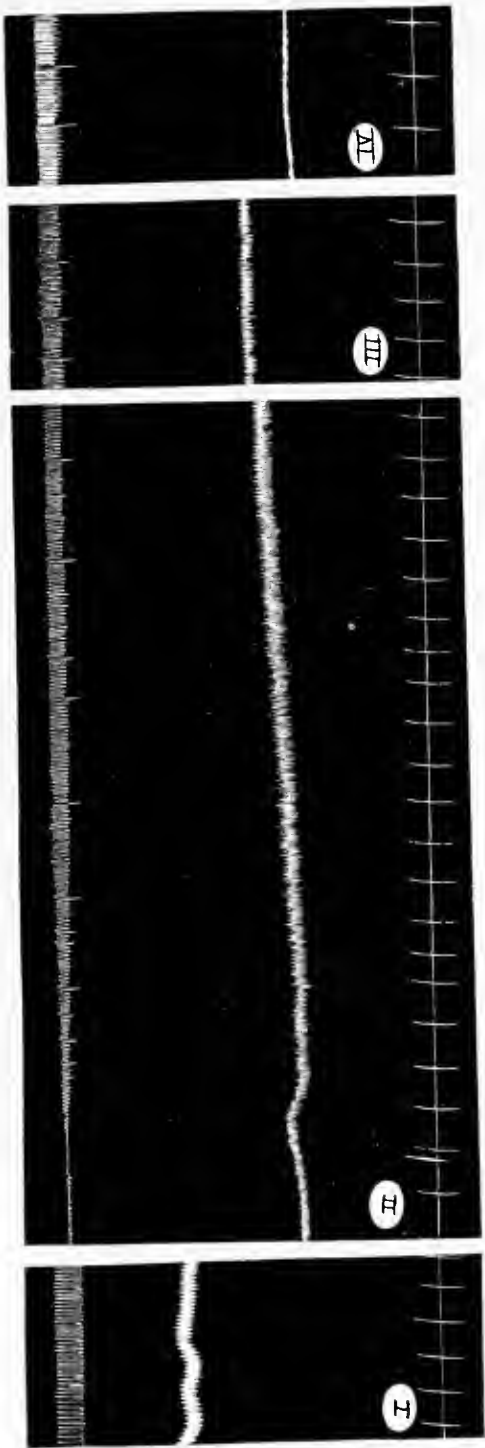


FIG. 2.

(Drinker: Useful heart method.)





## AN IMPROVED ANAEROBE JAR.

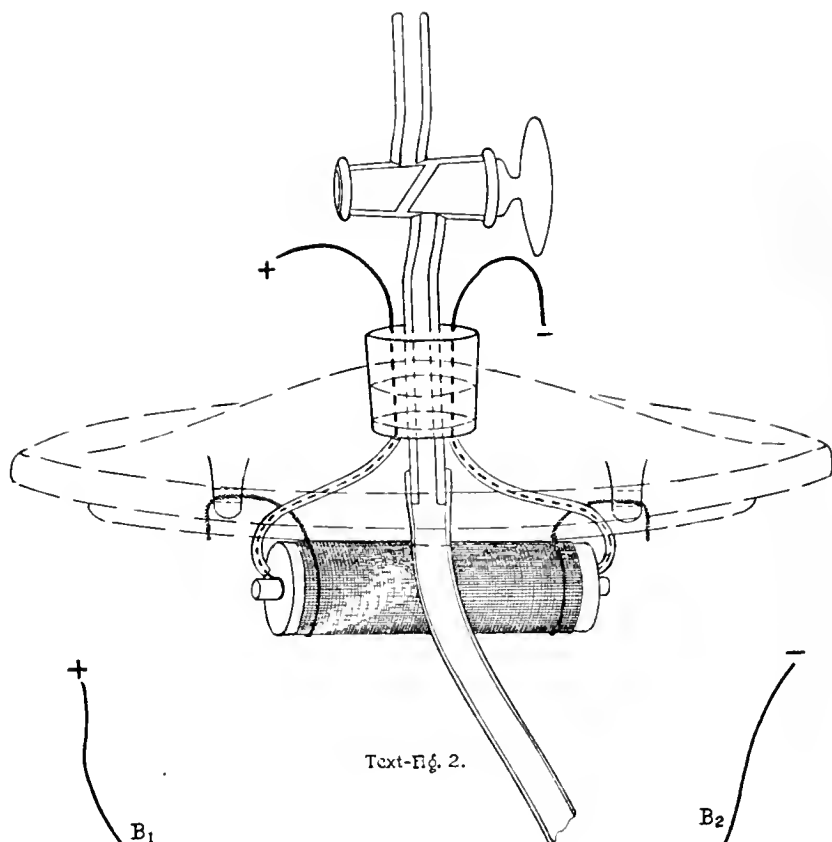
By J. HOWARD BROWN, PH.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

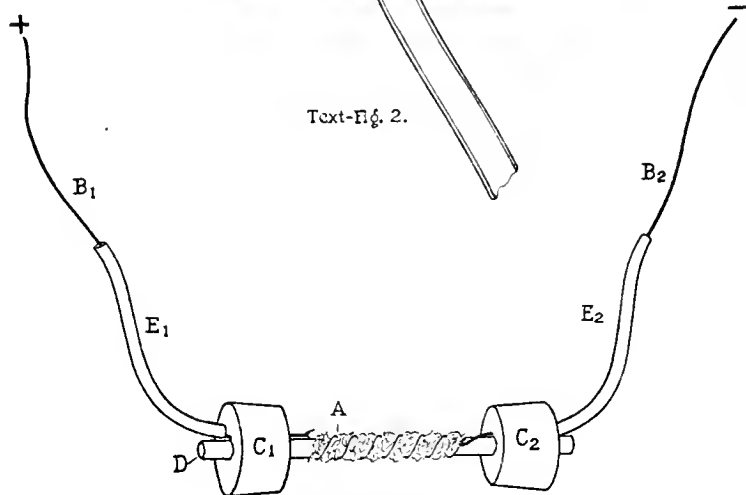
(Received for publication, February 23, 1921.)

Laidlaw (1) introduced the use of platinized carbon as a catalyzer for the combustion of oxygen and hydrogen to produce conditions favorable to the growth of anaerobic bacteria. His method was adapted for use with single tube cultures only. McIntosh and Fildes (2, 3) utilized the principle for the growth of anaerobes in jars. Their catalyzer consists of a small amount of platinized or palladinized asbestos wrapped in a piece of wire gauze and suspended from the lid of the jar. Hydrogen is introduced into the jar through a stop-cock in the lid. The method of McIntosh and Fildes was modified by W. G. Smillie (4) who enclosed the platinized asbestos in a small perforated glass bulb attached to the end of the tube through which the hydrogen is introduced into the jar, the hydrogen thus entering the jar through the asbestos. Fildes (5) has published a very useful review of these and other methods for the growth of anaerobic bacteria.

In the above mentioned methods the catalyzer must be heated in a flame, the tube or jar quickly closed, and the hydrogen introduced before the catalyzer has cooled, or else combustion does not occur. When the reaction has once started, however, the heat of combustion is sufficient to carry it to completion. There may, however, remain in the recesses of the jar or tubes or within the media traces of oxygen which diffuse out into the jar very slowly after the catalyzer has cooled and combustion has ceased. It is to be noted that Smillie's jar provided for intimate contact of the hydrogen but not of the oxygen with the catalyzer. He may have experienced some difficulty in this regard for in describing another form of apparatus he says: "The following method was devised to remove all the oxygen." The platinized asbestos was wrapped in a coil of fine nichrome wire the ends of which were joined to larger wires which passed upward through a rubber stopper. Hydrogen was introduced into a tube of inoculated solid medium and the rubber stopper inserted. The catalyzer was heated by passing an electric current through the coil surrounding it. Smillie points out that the tube may be set aside for a while and the asbestos reheated to ignite any residual oxygen. Apparently he got the most perfect anaerobic conditions with this apparatus. He did not utilize an electrically heated coil in jars in which many cultures could be enclosed, probably because of the danger of explosion when larger volumes of hydrogen and oxygen are used.



Text-Fig. 2.



Text-Fig. 1.

TEXT-FIG. 1. Showing the construction of the catalyzer coil. *A*, the fine nichrome wire coiled about the platinized or palladinized asbestos. *B*<sub>1</sub> and *B*<sub>2</sub>, larger copper wires joined to the ends of the nichrome wire. *C*<sub>1</sub> and *C*<sub>2</sub>, rubber stoppers. *D*, the core of glass tubing. *E*<sub>1</sub> and *E*<sub>2</sub>, small rubber tubing serving as insulation.

TEXT-FIG. 2. Showing the coil enclosed by fine copper wire gauze and in position beneath the lid of the anaerobe jar.

In the apparatus to be described the danger of explosion is eliminated by completely enclosing the asbestos and coil of nichrome wire in a copper wire screen which does not come in contact with the coil or asbestos at any point. This introduces the principle of the Davy safety lamp. The apparatus is illustrated in Text-figs. 1 and 2, and is made as follows:

A piece of fine nichrome wire (B. and S. gauge No. 28) (Text-fig. 1, *A*) is joined at each end with pieces of larger copper wire (*B*<sub>1</sub> and *B*<sub>2</sub>). One of the copper wires (*B*<sub>1</sub>) is inserted through a No. 1 one-hole rubber stopper (*C*<sub>1</sub>) and beside the wire is inserted also the end of a short piece of small glass tubing (*D*) holding the wire in the position shown in Text-fig. 1. Some palladinized asbestos is spread out onto a square of lens paper. This is then wrapped about the center of the glass tube and held in place by coiling the nichrome wire around it. The other copper wire (*B*<sub>2</sub>) is then passed through another rubber stopper (*C*<sub>2</sub>) which is placed over the other end of the glass tube. Pieces of small rubber tubing provide insulation for the copper wires at *E*<sub>1</sub> and *E*<sub>2</sub>. A piece of fine copper wire gauze is rolled around the entire core and held in place by wires twisted about the stoppers at each end of the gauze. The twisted ends of these wires serve to fasten the cell to the lid of the jar as shown in Text-fig. 2. An ordinary round museum specimen jar is used. A one-hole rubber stopper carrying a glass stop-cock is inserted into a hole bored in the lid of the jar. The two copper wires coming from the coil are run up through the rubber stopper on either side of the stop-cock. This is easily done by sticking a large hypodermic needle down through the stopper, running the copper wire up through the bore of the needle and then withdrawing the needle, leaving the wire in place. In time leaks are likely to appear around the stopper, the wires, or the stop-cock, so that cement or sealing wax should be placed over and around the rubber stopper. From the lower end of the stop-cock a rubber tube leads to the bottom of the jar to insure a good mixture of the oxygen with the hydrogen entering the jar.

In use, the lid is clamped down onto the jar of cultures over a gasket of "plasticine" modeling clay. An electric current is connected with the two protruding copper wires and hydrogen is run into the jar through the stop-cock under pressure of about 5 pounds.

Combustion is soon manifested by the collection of moisture inside the jar and by the lid becoming quite warm. This is allowed to continue until the flow of hydrogen ceases, as may be detected by observing no more bubbles in the wash bottles of the hydrogen apparatus. 20 or 30 minutes are usually sufficient, after which the stop-cock is closed, the electric current disconnected, and the jar incubated. At any time during incubation the electric current may again be passed through the coil to consume any residual oxygen. Sufficient hydrogen for this purpose will remain in the jar. A tube of gelatin tinted with methylene blue and decolorized in boiling water just before being sealed within the jar serves as an indicator of the presence or absence of oxygen.

An electric light current of 110 volts reduced by passage through a 60 watt Mazda lamp has been used for heating the coil. Very little hydrogen is required since none is passed into and out of the jar as is the case with the Novy jar.

A number of coils and jars as described have been in use for several months. There have been no accidents due to explosion such as have been known to occur with other jars employing the combustion principle. Whenever perfect anaerobic conditions have not been attained the failure has been due to small leaks in the stopper or stop-cock. The coils have shown no deterioration with use or age. The bit of lens paper used to hold the asbestos in place when the nichrome wire is being coiled about it is burned off with the first passage of the electric current. Similar cells in which the hydrogen was introduced through the asbestos into the cell were made but found to possess no advantage over the one described.

#### SUMMARY.

There has been described a modification of the anaerobe jars of McIntosh and Fildes and of Smillie in which the oxygen is consumed by combustion with hydrogen under the catalytic action of platinized or palladinized asbestos.

The special advantages of the apparatus described reside in its greater safety and in the fact that the catalyzer is heated electrically after the jar is closed and may be reheated at any time during incubation without opening the jar.

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2. McIntosh, J., and Fildes, P., A new method for the isolation and cultivation of anaerobic microorganisms, *Lancet*, 1916, i, 768.
3. McIntosh, J., and Fildes, P., Nouvelle méthode d'isolement et de culture pour les microbes anaérobies, *Compt. rend. Soc. biol.*, 1916, lxxix, 293.
4. Smillie, W. G., New anaerobic methods, *J. Exp. Med.*, 1917, xxvi, 59.
5. Fildes, P., Methods for cultivating the anaerobic bacteria, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 12*, 1917, 59.



## ETIOLOGY OF YELLOW FEVER.

### XIII. BEHAVIOR OF THE HEART IN THE EXPERIMENTAL INFECTION OF GUINEA PIGS AND MONKEYS WITH *LEPTOSPIRA ICTEROIDES* AND *LEPTOSPIRA ICTEROHÆMORRHAGÆ*.

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PLATES 85 AND 86.

(Received for publication, March 9, 1921.)

It is well known that in patients suffering from yellow fever the rate of the heart has a tendency to be slow. This phenomenon was regarded by Faget,<sup>1</sup> who was among the first to call attention to it, and by Touatre<sup>2</sup> as pathognomonic in this disease, especially in its early stage. In the second stage, the rate falls still further. It may be as low as 30 or 40 per minute, and may continue slow during convalescence. In fatal cases in the final stage it may be either rapid or slow. In the curves of patients published by Noguchi<sup>3</sup> and by Elliott,<sup>4</sup> these relations are seen. Sarti<sup>5</sup> gives, however, high rates for ten of the eleven cases he reports. It is said that death is seldom due to heart failure. At autopsy, lesions of the heart are not constantly found, except perhaps the degenerative changes and frequent

<sup>1</sup> Faget, J. C., *Monographie sur le type et la spécificité de la fièvre jaune établie avec l'aide de la montre et du thermomètre*, Paris, 1875.

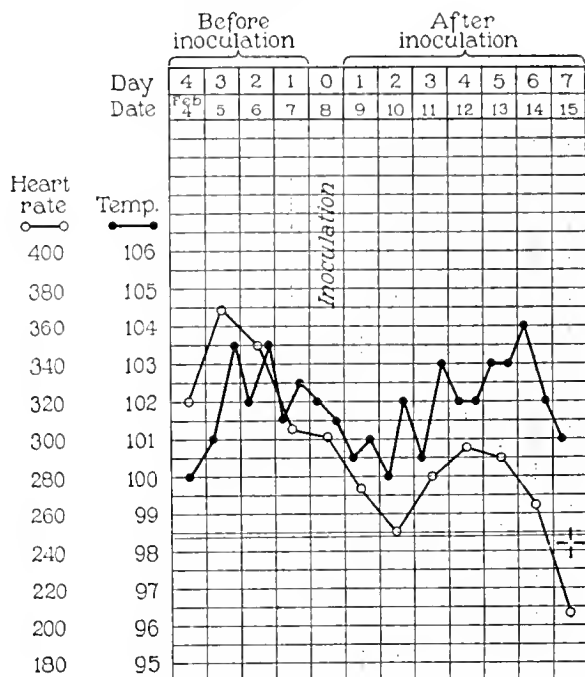
<sup>2</sup> Touatre, J., *Yellow fever; clinical notes*, translated by Chassaignac, C., New Orleans, 1898.

<sup>3</sup> Noguchi, H., *Etiology of yellow fever. I. Symptomatology and pathological findings of the yellow fever prevalent in Guayaquil*, *J. Exp. Med.*, 1919, xxix, 547.

<sup>4</sup> Elliott, C. A., *A clinical study of yellow fever. Observations made in Guayaquil, Ecuador, in 1918*, *Arch. Int. Med.*, 1920, xxv, 174.

<sup>5</sup> Sarti, J., *Contribucion al estudio de la fiebre amarilla*, Tesis, Guatemala, 1919.

endocardiac or pericardiac ecchymoses associated with fever. Recent studies on infectious jaundice<sup>6</sup> indicate, however, that in that disease also the pulse rate is usually slow in proportion to the temperature; one case is reported in which the pulse fell as low as 38. This holds true even in cases without jaundice.



TEXT-FIG. 1. Temperature and heart rate curves of Guinea Pig 3, inoculated with *Leptospira icteroides*.

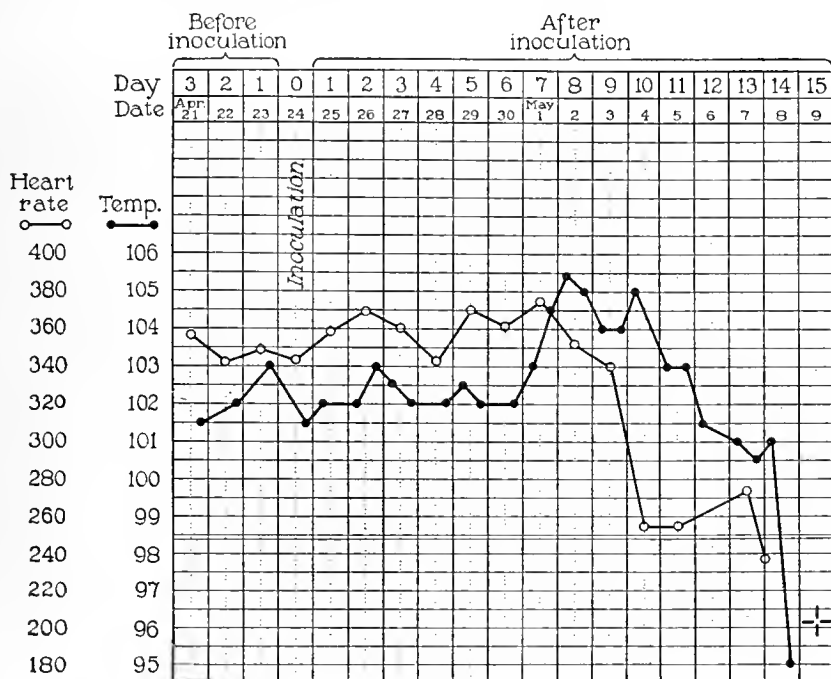
Since the successful transmission of yellow fever to animals, observations have been made of the rate and of the behavior of the heart in animals experimentally infected with *Leptospira icteroides* from yellow fever cases. Records were taken electrocardiographically.

<sup>6</sup> Stokes, A., Ryle, J. A., and Tytler, W. H., Weil's disease (spirochætosis ictero-hæmorrhagica) in the British Army in Flanders, *Lancet*, 1917, i, 142. Ryle, J. A., Spirochætosis icterohæmorrhagica (formerly known as Weil's disease). A clinical analysis of fifty-five cases, *Quart. J. Med.*, 1921, xiv, 139.



By this method the rate can be accurately counted and the mechanism of slowing, if it occurs, can be analyzed. For comparison with the animals inoculated with *Leptospira icteroides* (yellow fever) a certain number of others infected with *Leptospira icterohæmorrhagiae* (infectious jaundice) were included in the study.

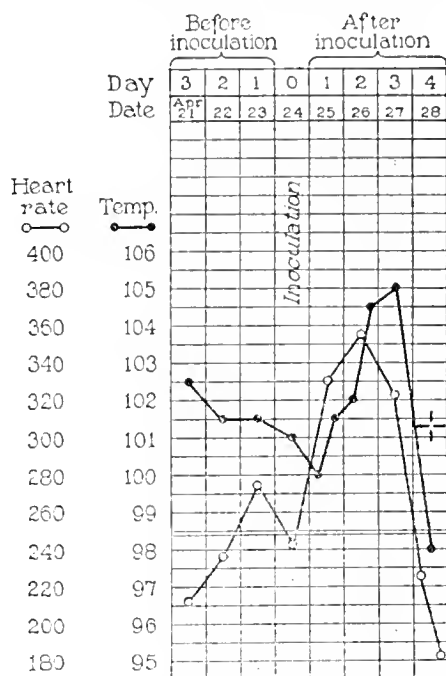
Electrocardiograms were taken either for 1, 3, or 4 days before the day of inoculation. Exception to this procedure occurred in Guinea



TEXT-FIG. 2. Temperature and heart rate curves of Guinea Pig 12, inoculated with *Leptospira icterohæmorrhagiae*.

Figs 4 and 6 and Monkey 3. The cardiac rate before inoculation of the guinea pigs was below 300 per minute three times in Guinea Pig 1, twice in Guinea Pig 2, and once in Guinea Pig 10. In the other nine guinea pigs it was always above 300, the range being from 302 to 371. In the monkeys the rate was above 400, except once in Monkey 2, and once in Monkey 5. The rate otherwise was above 400, the range being from 401 to 442.

In eleven animals there occurred a fairly consistent fall in rate either immediately after inoculation (Guinea Pigs 3, 7, and 9 and Monkey 2) (Text-fig. 1) or after a delay (Guinea Pigs 2, 4, 5, 8, and 12 and Monkeys 3 and 4) (Text-fig. 2). In several instances (Guinea Pigs 2, 4, 7, and 9) a rise occurred in the course of the disease. In each instance, however, there was a striking fall on the day of death, or during the days immediately preceding death. Guinea Pig 2



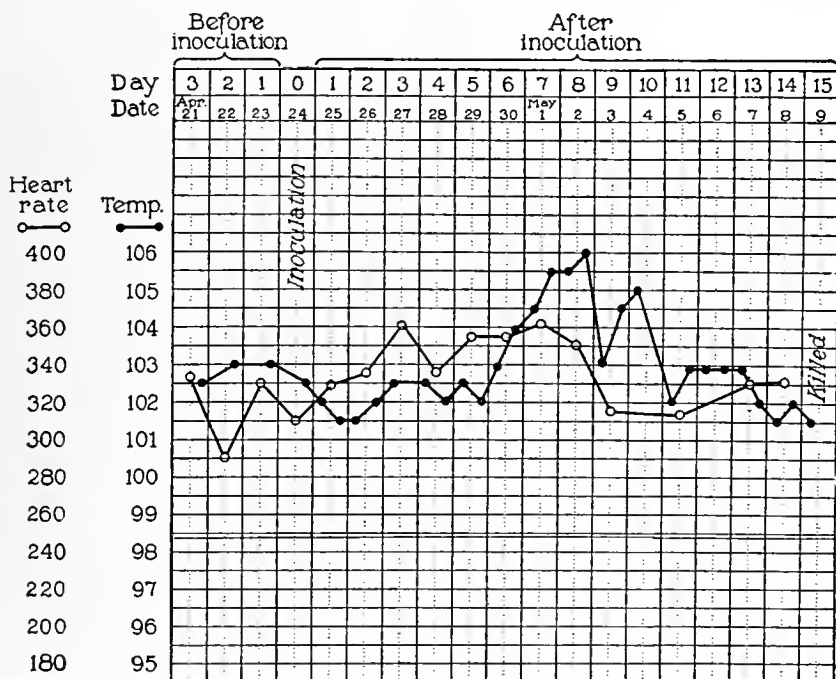
TEXT-FIG. 3. Temperature and heart rate curves of Guinea Pig 1, inoculated with *Leptospira icteroides* but with negative results.

appears to be an exception, but this is one of the animals in which low initial rates were recorded. The animals inoculated with *Leptospira icteroides* reacted, then, with a fall in rate. Those inoculated with *Leptospira icterohæmorrhagiæ* behaved in a similar manner (Table I).

In Guinea Pigs 1 (Text-fig. 3) and 6 the rates before inoculation were low, rose abruptly to high levels after inoculation, and then

fell. Guinea Pig 1 was inoculated with *Leptospira icteroides*, but died of a complicating peritonitis. Guinea Pig 6 ran an atypical course and died perhaps of a secondary infection.

This account leaves for consideration four animals, Monkeys 1 and 5 and Guinea Pigs 10 (Text-fig. 4) and 11. The monkeys did not develop disease of a nature that could be regarded as characteristic and are therefore offered as controls. They showed no striking



TEXT-FIG. 4. Temperature and heart rate curves of Guinea Pig 10, inoculated with *Leptospira icterohæmorrhagiæ* but with negative results.

change in rate. Like observations are afforded by Guinea Pigs 10 and 11. They were killed after 14 days. The course of the disease in them was mild and the lesions were atypical. They are, therefore, also to be regarded as controls.

*Mechanism of the Heart Beat.*—During the control period preceding inoculation in the *icterohæmorrhagiæ* group, a single animal, Guinea Pig 12, showed an irregularity of the cardiac mechanism on one

TABLE I.  
Records of the Pulse Rate of Animals Inoculated with *Leptospira icteroides* and *Leptospira icterohaemorrhagiae*.

Animal No.	Rate.																				Fall in rate.*	Irregularity.	Result.
	Days before inoculation.				Day of inoculation.	Days after inoculation.																	
	4	3	2	1		Average.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
G. P. † 1		211	235	275	240	243	330	355	322	181	D. †										-59	-	Died in 4 days. Negative: <i>L. icteroides</i> G§ 5. Peritonitis. (Text-fig. 3.)
" 2		221	256	335	271	292	340	331	350	278	286	315	D.								+44	-	Died in 7 days. Positive: <i>L. icteroides</i> G§ 5. Fever 3rd, 4th, 5th, and 6th days; peritonitis.
" 3	320	369	350	305	338	300	273	250	280	295	290	265	208	D.							-130	+	Died in 7 days. Typical: <i>L. icteroides</i> G§ 5. Fever 4th, 5th, and 6th days (Text-fig. 1).
" 4							266	275	303	330		265	233	D.							-33	+	Died in 6 days. Typical: <i>L. icteroides</i> G§ 6. Fever 3rd, 4th, 5th, and 6th days.
" 5	345	371	361	345	355	336	318	319	340	327	322	301	320	298	323	292	304				-51	-	Recovered in 10 days. Mild: <i>L. icteroides</i> G§ 6. Fever 5th day; mild.
" 6							211	458	375	311	257	D.									+46	-	Died in 4 days. Atypical: <i>L. icteroides</i> G§ 6. Fever 4th day; perhaps secondary infection

Died in 4 days.  
Negative: *L. icteroides* G§ 5.  
Peritonitis. (Text-fig. 3.)  
Died in 7 days.  
Positive: *L. icteroides* G§ 5.  
Fever 3rd, 4th, 5th, and 6th days;  
peritonitis.  
Died in 7 days.  
Typical: *L. icteroides* G§ 5.  
Fever 4th, 5th, and 6th days  
(Text-fig. 1).  
Died in 6 days.  
Typical: *L. icteroides* G§ 6.  
Fever 3rd, 4th, 5th, and 6th days.  
Recovered in 10 days.  
Mild: *L. icteroides* G§ 6.  
Fever 5th day; mild.  
Died in 4 days.  
Atypical: *L. icteroides* G§ 6.  
Fever 4th day; perhaps secondary  
infection.



TABLE 1—*Concluded.*

Animal No.	Rate.																			Fall in rate.	Irregularity.	Result.	
	Days before inoculation.				Day of inoculation.	Days after inoculation.																	
	4	3	2	1		Average.	1	2	3	4	5	6	7	8	9	10	11	12	13	14			15
G. P. 10	333	289	332	318	309	327	334	363	335	357	355	362	352	313	314	330	331				+13	—	Killed in 14 days. Probably negative or very mild: <i>L. icterohæmorrhagiæ</i> (French). Fever 7th to 10th days; no jaundice; no hemorrhage; no lesions (Text-fig. 4).
" " 11	360	340	360	353	361	360	376	363	350	371	367	362	342	306	320	327	328				—25	—	Killed in 14 days. Mild: <i>L. icterohæmorrhagiæ</i> (French). Fever 7th to 14th days; intercurrent infection; no jaundice; no hemorrhage; no lesions.
" " 12	358	343	347	349	345	360	370	363	344	370	363	374	356	340	254	276	238	D.			—111	+	Died in 14 days. Atypical: <i>L. icterohæmorrhagiæ</i> (French). Fever 7th to 10th days; incubation 6½ days (Text-fig. 2). Died in 4 days.
M. 5	436	436	372	415	432	415	431	392	370	D.											—45	—	Negative: <i>L. icterohæmorrhagiæ</i> (French). Doubtful; irregular fever; slight hemorrhage in lungs; no jaundice.

occasion. During the 14 days following, with two exceptions, curves were taken daily. A recurrence of the arrhythmia was not seen. During the course of the disease nothing remarkable in the behavior of the heart was recorded, although curves of the animals in this group which died were taken on the day of death (Guinea Pig 12 and Monkey 5) and on the day preceding (Monkey 4).

Of the yellow fever group, no irregularity was seen in any animal in the period preceding inoculation. A single guinea pig, No. 7, on the 2nd day afterward, showed an arrhythmia (premature ventricular contractions). It died 5 days later but the irregularity did not recur. In four other animals, irregularities took place (Guinea Pigs 3 and 4 and Monkeys 2 and 3), but in two of them (Guinea Pigs 3 and 4) they occurred on the day of death, indeed during the terminal period. At this period Guinea Pig 3 showed heart block (Fig. 1). Monkey 2 (Fig. 2) began to show changes in the electrocardiogram on the day before death. The alterations are not different from those which have been described in connection with the death of the heart in other diseases. There was slowing of the auricles and the whole heart, and increase in the length of the auriculoventricular interval in Guinea Pig 3 and Monkey 2 (Fig. 2) and lengthened conduction time leading to block in Guinea Pig 3. In only two of the animals, Monkeys 2 and 3 (Fig. 3), did changes occur before the day of death. In both the change was detectable 2 days before death. Both showed slowing of the rate and alteration in the form of the ventricular complex in the electrocardiogram. An S wave not previously present appeared, and the T wave increased in size (Fig. 2).

It may be said, then, that the mechanism of slowing in all cases until the day of death was of a simple variety—a slowing of the whole heart, depending on the sinus, the pacemaker of the heart. On the day of death, although increased length of the auriculoventricular interval occurred, it led to heart block only once. Changes in the form of the electrocardiogram were seen twice (Monkeys 2 and 3, Fig. 3) in the course of the disease 2 days before death. In curves that were obtained during death of the heart changes were seen which resemble those usually found at this time.

## CONCLUSIONS.

1. Slowing of the heart occurred in monkeys and guinea pigs during the febrile period of the experimental infection due to *Leptospira icteroides*. A similar reaction took place in animals inoculated with *Leptospira icterohæmorrhagiæ*.

2. The mechanism of slowing was usually due to slowing of the whole heart.

3. Once incomplete heart block was seen. Changes in the ventricular complex occurred four times.

## EXPLANATION OF PLATES.

In the curves divisions of the abscissæ equal 0.04 second, divisions of the ordinates 0.1 millivolt. The electrodes were placed on the right fore and the left hind legs.

## PLATE 85.

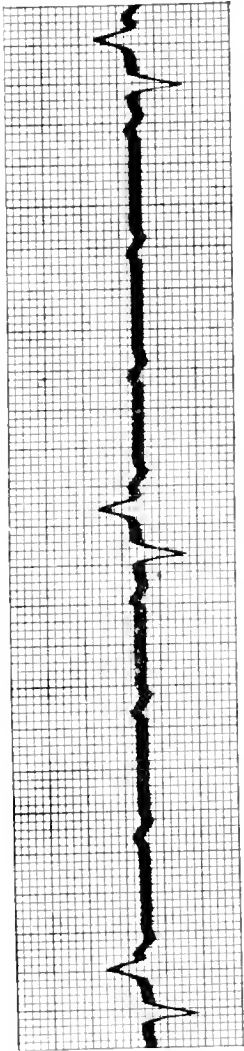
FIG. 1, *a* and *b*. Guinea Pig 3. (*a*) February 4, 1919. Control curve. (*b*) February 15. Auriculoventricular dissociation and a marked change in the ventricular complex are seen.

FIG. 2, *a* and *b*. Monkey 2. (*a*) April 21, 1919. Control curve. (*b*) April 30. Slowing, increase in height of R, S, and T waves, and increased auriculoventricular interval are seen.

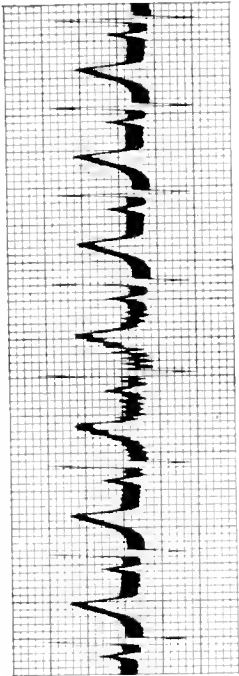
## PLATE 86.

FIG. 3, *a* to *g*. Monkey 3. (*a*) April 7, 1919. Control curve. (*b*) April 13. Day before death. S wave is increased. (*c*) April 14. Day of death. The R, S, and T waves are increased. The auriculoventricular interval is lengthened. (*d*, *e*, *f*, *g*) show increasing alteration in both auricular and ventricular parts of the curve. The ventricular alterations resemble those seen in dying hearts.

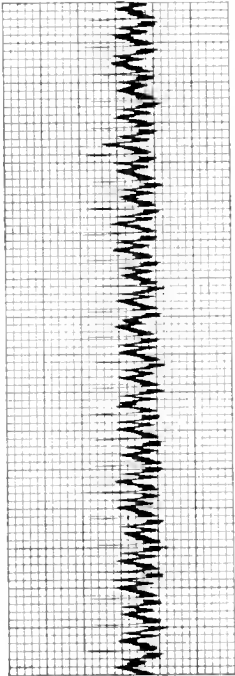




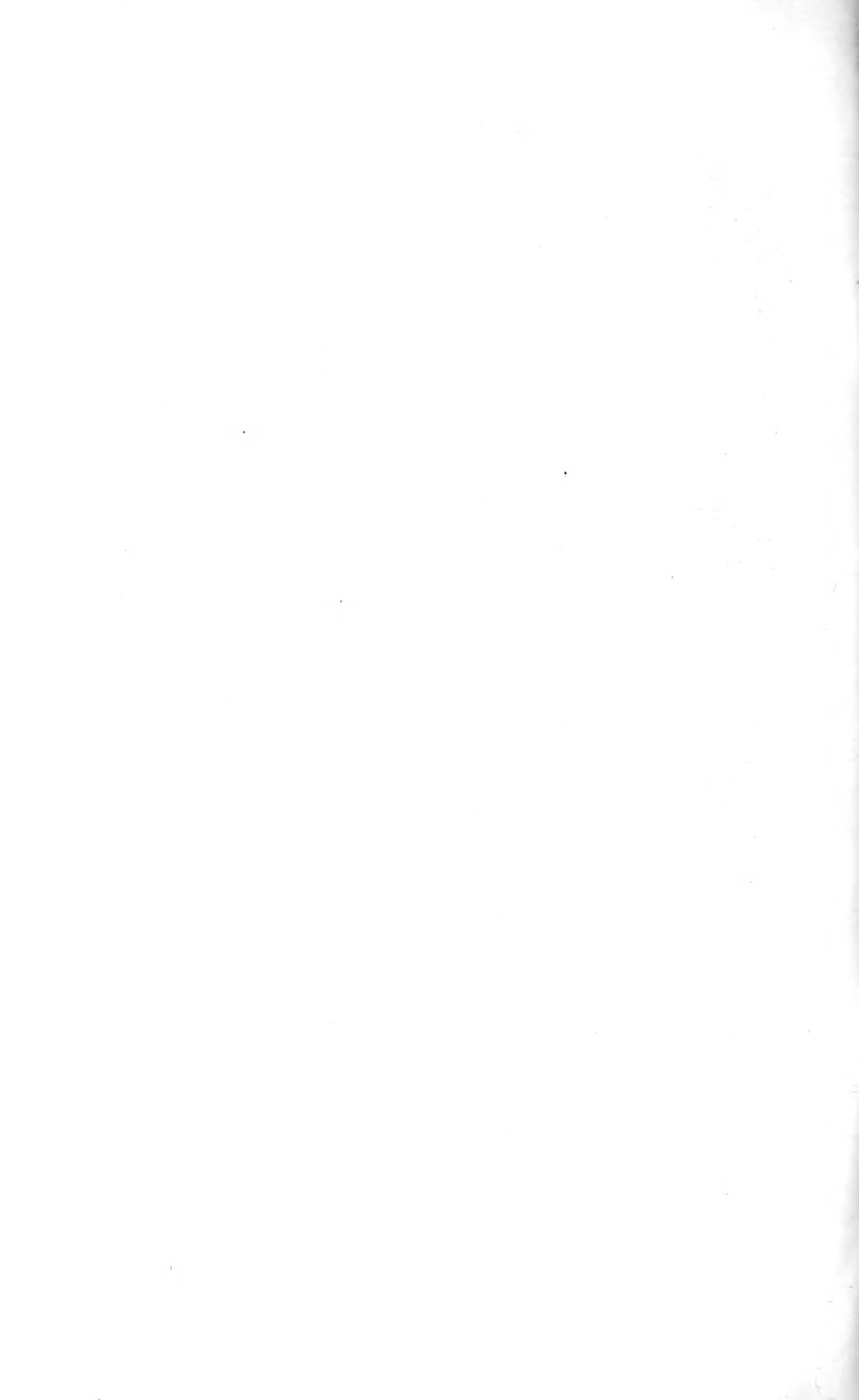
1 b

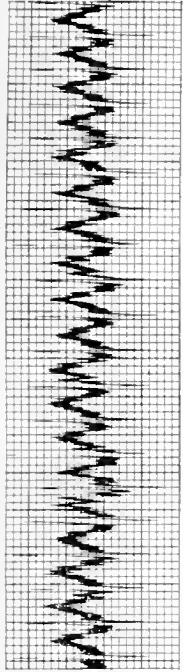


2 b

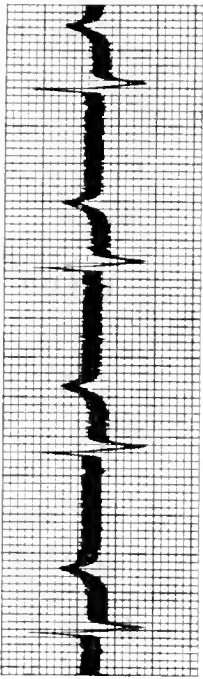


(Cohn and Noguchi: Etiology of yellow fever. XIII.)

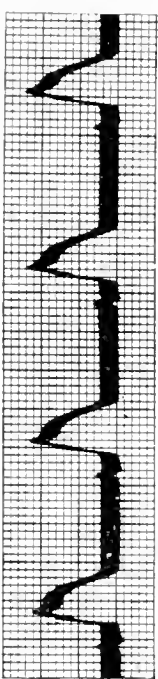




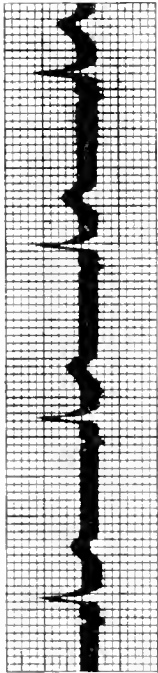
3 a



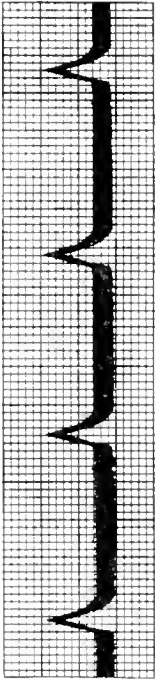
3 b



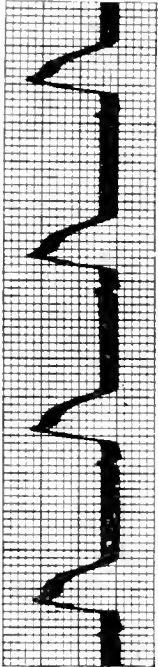
3 c



3 d



3 e



3 f

3 g



# CHEMOTHERAPEUTIC STUDIES WITH ETHYLHYDROCUPREINE HYDROCHLORIDE IN EXPERIMENTAL PNEUMOCOCCUS PLEURITIS.

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PLATES 87 TO 94.

(Received for publication, February 17, 1921.)

In previous experiments Kolmer and Idzumi (1) found that the subthecal injection of single doses of ethylhydrocupreine hydrochloride in amounts of 0.0005 gm. per kilo of weight had a distinctly beneficial effect on the course of acute suppurative meningitis in rabbits produced by Type I pneumococci of moderate virulence, when administered not later than 4 to 6 hours after infection; when given 24 hours or longer after the pneumococci, this effect was not apparent. In experiments consisting in the repeated subthecal injection of smaller doses of pneumococci followed in a few hours by ethylhydrocupreine, the protective and curative effects of the drug were more apparent.

Unquestionably, ethylhydrocupreine (optochin) hydrochloride shows marked pneumococcidal activity *in vitro* in menstrea of saline solution and in the presence of large amounts of protein, also some protective and curative properties in experimental pneumococcus bacteremias in mice. However, the drug is too toxic to permit the administration of sufficient amounts to individuals to influence materially the severity and mortality of lobar pneumonia, but experiments on pneumococcus meningitis have indicated that it may be possible to inject enough ethylhydrocupreine into closed sacs to raise the pneumococcidal activity of the exudates without danger of irritation of the serous membranes or the absorption of toxic amounts.

These results suggested similar experiments on acute suppurative pneumococcus pleuritis induced in guinea pigs and dogs as having a

possible value in the treatment of pneumococcus pleuritis with empyema developing during croupous pneumonia.

The purposes of the experiments were as follows: (1) to determine the pneumococidal activity of ethylhydrocupreine hydrochloride in pleural pus; (2) to determine the toxic effects of ethylhydrocupreine injected into the pleural sacs; (3) to produce experimentally suppurative pneumococcus pleuritis; and (4) to determine the curative effects of ethylhydrocupreine in experimental suppurative pneumococcus pleuritis.

*Pneumococidal Activity of Ethylhydrocupreine in Pleural Pus.*

Numerous experiments by Morgenroth and Levy (2), Moore (3), Solis-Cohen, Kolmer, Heist, and Steinfield (4), and others have

TABLE I.

*Bactericidal Effect of Ethylhydrocupreine on Type I Pneumococci in Pleural Pus.*

1 cc. of ethylhydrocupreine.	Pus.	Final dilution.	Results of subcultures after exposure at 37°C.			
			5 min.	30 min.	60 min.	120 min.
	cc.					
1:100	1	1:200	—*	—	—	—
1:500	1	1:1,000	+	—	—	—
1:1,000	1	1:2,000	+	—	—	—
1:2,000	1	1:4,000	+	+	+	+
1:3,000	1	1:6,000	+	+	+	+
Saline control.	1	—	+	+	+	+

\* — indicates that subcultures were sterile; + indicates living pneumococci.

attested to the high bacteriostatic and bactericidal activity of ethylhydrocupreine for pneumococci *in vitro* in menstrua of saline solution and of serum. Germicidal tests *in vitro* by Kolmer and Idzumi (1) with purulent spinal fluids containing virulent Type I and Type II pneumococci showed ethylhydrocupreine to be bactericidal in a final dilution as high as 1:4,000 when equal parts of spinal fluid and drug solution were brought together for 1 hour at 37°C.

In the present study similar experiments *in vitro* with thick creamy pleural pus obtained from a patient and containing Type I pneumococci showed that with equal parts of pus and 1:1,000 solution of ethylhydrocupreine in saline solution complete sterilization occurs

after about 30 minutes in a water bath at 37°C. (Table I). Films of this pus showed the presence of enormous numbers of pneumococci, most of which were extracellular.

Portions of the same pus were heavily inoculated with a laboratory culture of *Staphylococcus aureus* and the bactericidal tests were repeated; mixtures of equal parts of pus and 1:100 solution of ethylhydrocupreine (final dilution 1:200) showed no appreciable diminution of the staphylococci after exposures as long as 2 hours, although the pneumococci were destroyed. Numerous experiments of this kind by the investigators previously mentioned have shown the high selective bactericidal activity of ethylhydrocupreine for pneumococci.

TABLE II.

*Toxicity of Ethylhydrocupreine Hydrochloride Injected Intrapleurally in Guinea Pigs.*

Weight of guinea pig.	Amount of 1:100 solu- tion per 100 gm.	Amount of 1:100 solu- tion injected.	Amount of drug per kilo.	Amount of 1:100 solu- tion injected per kilo.	Result.
gm.	cc.	cc.	gm.	cc.	
360	1.0	3.6	0.100	10.0	Convulsions; died in 2½ min.
400	0.5	2.0	0.050	5.0	" " " 7 "
420	0.5	2.1	0.050	5.0	" " " few "
460	0.3	1.38	0.030	3.0	" " " " "
400	0.3	1.2	0.030	3.0	" " " " "
540	0.2	1.08	0.020	2.0	Nervous; coughing; lived.
400	0.2	0.8	0.020	2.0	Coughing; lived.
560	0.1	0.56	0.010	1.0	No symptoms; lived.
400	1.0	Control.	None.	10.0	Slight cough; "
	(water).			(water).	

*Toxicity of Ethylhydrocupreine Injected into the Pleural Sacs.*

Table II shows the results observed in an experiment consisting in the intrapleural injection of a 1:100 solution of ethylhydrocupreine into the pleural cavities of guinea pigs weighing from 350 to 400 gm.<sup>1</sup> The drug proved slightly more toxic when injected intrapleurally than when injected intravenously; this study has been extended with the use of other drugs and animals (5) with similar results.

<sup>1</sup> All injections were done under local anesthesia.

In each experiment the solutions were warmed and slowly injected; these are factors of importance in relation to toxicity. As shown in Table II the highest tolerated dose of ethylhydrocupreine hydrochloride for guinea pigs by the intrapleural route is between 0.2 and 0.3 cc. of a 1:100 solution per 100 gm. of weight, which is equivalent to 2 to 3 cc. or 0.02 to 0.03 gm. of the drug per kilo of body weight. In experiments on acute pleuritis 0.5 to 1 cc. of a 1:500 solution was injected into each pleural cavity of guinea pigs weighing from 350 to 500 gm. These amounts were two and a half to five times less than the toxic doses and produced no gross or histological evidences of irritation of the pleura as determined by necropsies and histological studies at varying intervals after the intrapleural injections (Fig. 1).

The slow injection of as much as 15 cc. per kilo of weight of a warm 1:500 solution of ethylhydrocupreine into the right pleural cavity of a normal dog produces no immediate symptoms, except possibly a few short coughs; this dose has produced no discernible evidences of toxicity. When examined at necropsy 24 to 72 hours after injection a small amount of fluid is found in the pleural cavity accompanied by slight hyperemic changes in the lung but no other gross changes. Histological examination usually reveals hyperemia of the lung with slight hyperemia and leucocytic infiltration of the pleura (Fig. 2). This amount of solution introduces as much as 0.030 gm. of the drug, corresponding to 900 cc., or 1.8 gm., per 60 kilos of weight and representing an amount near the highest tolerated dose.

In as far as volume of fluid injected into a pleural cavity is concerned, it would appear that guinea pigs and dogs show no immediate effects, except a few short coughs, or remote effects from the slow injection of as much as 5 to 10 cc. of warm sterile water or saline solution per kilo of weight; these amounts correspond to 300 to 600 cc. per 60 kilos, or the weight of a young adult person.

#### *Experimental Pneumococcus Pleuritis.*

Experiments were conducted with a Type I pneumococcus from the sputum of a case of croupous pneumonia of such virulence that 0.000001 cc. of a 24 hour dextrose blood broth culture was usually fatal for a white mouse about 36 hours after intraperitoneal injection.



For the purpose of producing experimental pleuritis, guinea pigs and dogs were chosen because of their higher natural immunity to the pneumococcus. Rabbits were liable to succumb with pneumococcus bacteremia after the intrapleural injection of amounts of pneumococcus culture too small for the production of well defined and extensive suppurative pleuritis. The majority of the experiments were conducted with guinea pigs.

*Pleuritis in Guinea Pigs.*—The intrapleural injection of guinea pigs with 0.5 cc. of 24 hour dextrose broth cultures of this strain usually killed in about 50 hours with extensive suppurative changes in both pleural sacs and the pericardium (Table III). The injections were

TABLE III.

*Production of Pleuritis in Guinea Pigs by the Intrapleural Injection of 24 Hour Dextrose Broth Cultures of Virulent Type I Pneumococci.*

Weight of guinea pig.	Amount of culture.	Duration of life.	Cultures of heart.	Result.
gm.	cc.	hrs.		
410	0.5	50	+	Bilateral suppurative pleuritis and pericarditis.
300	1.0	65	+	" " " "
380	1.0	24	+	" " " "
360	1.0	48	+	" " " "
380	2.0	50	+	" " " "
430	2.0 (broth control).	Lived.	0	Necropsy not performed.

\* + indicates pneumococci in films or cultures of blood or both; 0, not done.

made with aseptic technique in the anterior axillary line on the right side in the neighborhood of the sixth or seventh interspace, all injections being slowly given.

Necropsies usually disclosed pneumococci in the heart's blood and extensive suppurative changes in the pleuræ which were most marked on the right side but also involved the pleura on the left side and the external portions of the pericardium (Fig. 3). Each pleural sac usually showed the presence of a plastic exudate of a greenish yellow color; films showed large numbers of polymorphonuclear pus cells

and very large numbers of extracellular diplococci (Fig. 4). Sections of the visceral and parietal portions of the pleura showed the histological changes of acute suppurative pleuritis, with very large numbers of polymorphonuclear leucocytes and fibrin deposits (Figs. 5 to 9). As a rule, sections of the visceral portions of the pleura showed more extensive changes than sections of the parietal portions. Animals which survived for 5 days or were permitted to live for longer periods of time showed evidences of beginning organization of the inflammatory exudate (Fig. 7) or well marked fibrous tissue thickening of the visceral and parietal layers of the pleura, particularly of the former (Fig. 10).

Generally the lungs on both sides showed inflammatory changes. At necropsy portions were usually dark red in color and failed to collapse. Sections of these areas showed intense hyperemia and serous exudation (Figs. 11 and 12); occasionally the alveoli contained numerous polymorphonuclear leucocytes and alveolar epithelium. These changes were most marked just beneath the visceral pleura and in the areas of both lungs which showed the maximum macroscopic changes. Not infrequently the pleuritis was most severe over the anterior portions of both lungs and pericardium and at the bases of the lungs in proximity to the diaphragm.

Controls injected intrapleurally with relatively large amounts (up to 20 cc. per kilo) of sterile 0.1 per cent dextrose blood broth lived indefinitely. Necropsies performed at intervals never showed suppurative changes; at most small amounts of blood-stained serous fluid were found on the injected side after 24 hours. Animals necropsied several days after injection showed normal sacs, except occasionally for a few fine adhesions between the visceral and parietal portions of the pleura in the immediate neighborhood of the sites of injection. Since the broth produced at most a very slight localized pleuritis, we used 24 hour broth cultures of the pneumococcus instead of cocci grown on solid media or obtained by centrifugation of broth cultures and suspension in saline solution. The slight pyogenic activity of the broth alone was considered an aid in the production of a severe form of suppurative pleuritis.

*Pleuritis in Dogs.*—With dogs weighing about 5 kilos acute pleuritis terminating fatally in about 3 days was produced by injecting 15 cc.

of a 24 hour broth culture reinforced by washing into the broth the cocci from ten slants of 24 hour growths on blood agar in tubes of the usual size. Within 24 hours the animals were sick with increased rectal temperatures and leucocytosis. Necropsies on or about the 3rd day after injection showed large amounts of thin bloody exudate (210 cc. in one instance) which deposited a heavy creamy layer of pus upon standing. Films of this exudate showed polymorphonuclear leucocytes, erythrocytes, and large numbers of extracellular pneumococci. Cultures of the blood from the heart usually showed the presence of pneumococci. The pleuritis was generally much milder than that observed in guinea pigs; the pleural surfaces were dull red and covered with flecks of fibrinous exudate but were generally free of the massive exudates developing among guinea pigs. Furthermore, there was usually no involvement of the pleura on the opposite side or of the pericardium, except the portion in direct relation with the pleura on the right side.

Sections of the pleura and lung showed acute pleuritis with well marked pulmonary congestion and edema (Fig. 11) and infiltration of the pleura with polymorphonuclear leucocytes.

*Influence of Intrapleural Injections of Ethylhydrocupreine upon Pneumococcus Pleuritis.*

*Treatment with Single Doses of Ethylhydrocupreine Injected into the Right Pleural Sac.*—In these experiments guinea pigs weighing from 350 to 500 gm. were employed. Each animal was infected by injecting 1 cc. of a 24 hour dextrose broth culture into the right pleural sac. The ethylhydrocupreine<sup>2</sup> was prepared in a 1:500 dilution in sterile saline solution and used in a dose of 1 cc. for guinea pigs of the weight given above. This dose is approximately 2.5 cc. of the solution, or 0.005 gm. of the drug, per kilo of weight. The ethylhydrocupreine was warmed to 37°C. and slowly injected once into the right pleural sac immediately after inoculation and at intervals varying from 4 to 48 hours after infection. Controls with culture alone and drug alone were included in each experiment. Necropsies were conducted aseptically for the purpose of securing films and cultures of the heart's blood and exudates; sections of the visceral and parietal pleurae and lungs of each animal were prepared.

<sup>2</sup> Prepared by Zimmer and Company, Frankfort-on-the-Main.

In the first experiments, in which ethylhydrocupreine was injected into the right pleural sac only, there was a slight influence upon the infection as determined by the duration of life of the experimental animals. All animals died and usually showed acute suppurative pleuritis on both sides, which involved the pericardium and resembled that of the untreated controls. The results of one of the experiments are shown in Table IV.

TABLE IV.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1:500 Ethylhydrocupreine into the Right Pleural Sac\* at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.‡									
<i>hrs.</i>										
Immediately.	Lived about 48 hrs.; acute suppurative bilateral pleuritis and pericarditis.									
4	"	"	36	"	"	"	"	"	"	"
24	"	"	52	"	"	"	"	"	"	"
48	"	"	72	"	"	"	"	"	"	"
Culture alone (control).	Died in	48	"	"	"	"	"	"	"	"
Drug alone (control).	Lived.									

\* This is a dose equivalent to about 2.5 cc. per kilo.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ All animals except the drug control showed pneumococci in the heart's blood and in the pleural exudates.

*Treatment with Single Doses of Ethylhydrocupreine Injected into Both Pleural Sacs.*—On account of the development of pleuritis on the left side and of pericarditis following the injection of pneumococci into the right pleural sac, subsequent experiments with guinea pigs were performed in which one dose of 1 cc. of 1:500 ethylhydrocupreine was injected into each pleural sac immediately after infection and at intervals of 4 to 48 hours after infection. Controls with the culture alone and drug alone were included in each experiment.

The results of two experiments are shown in Tables V and VI. In nearly all instances the severity and extent of the local inflammatory

TABLE V.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1:500 Ethylhydrocupreine into Each Pleural Sac\* at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.‡
<i>hrs.</i>	
Immediately.	Lived.
4	" 48 hrs.; acute bilateral suppurative pleuritis and pericarditis.
24	"
48	" about 49 hrs.;§ acute bilateral suppurative pleuritis and pericarditis.
Culture alone (control).	" " 48 " " " " " " " "
Drug alone (control).	"

\* This is a dose equivalent to about 2.5 cc. per kilo in each sac.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ All animals necropsied showed pneumococci in the heart's blood and in the pleural exudates.

§ The animal was dying when injected with optochin; it died within 1 hour after injection.

TABLE VI.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 1 Cc. of 1:500 Ethylhydrocupreine into Each Pleural Sac\* at Varying Intervals after Infection.†*

Interval between time of infection and injection of drug.	Result.
<i>hrs.</i>	
Immediately.	Lived.
2	" 4 days; serous exudate with few plastic adhesions.
4	" 8 " no exudate; areas of consolidation in both lungs.
24	"
Culture alone (control).	" 2 days; acute bilateral suppurative pleuritis and pericarditis.
Drug alone (control).	"

\* This is a dose equivalent to about 2.5 cc. per kilo in each sac.

† Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

changes in the pleuræ and pericardium and the duration of life of the animals were greatly influenced by the drug when the latter was administered within 24 hours after infection (Figs. 13 and 14). Untreated controls always showed extensive pleuritis and pericarditis at the end of 24 hours and usually succumbed within 48 hours (Fig. 3). In practically all instances when drug immediately followed culture the animals showed no signs of illness. Some were necropsied at intervals for histological study and showed no pneumococci in the heart or pleural sacs and no gross evidences of infection (Fig. 15) although sections of the visceral pleura sometimes showed slight degrees of leucocytic infiltration.

When the drug was administered 2 to 24 hours after injection the results were more irregular; that is, sometimes an animal that received the drug 24 hours after infection lived longer and showed less inflammatory reaction than animals that received the drug 2 to 4 hours after infection. In the majority of animals, however, the drug appeared to modify the degree and extent of pleuritis and pericarditis, unless treatment was delayed too long; *i.e.*, if 48 hours were allowed to elapse between the time of intrapleural injection of 1 cc. of the culture and the injection of 1 cc. of 1:500 ethylhydrocupreine hydrochloride into each pleural sac. When treatment was delayed for 48 hours the guinea pigs were usually very sick and not infrequently died within a few minutes after the intrapleural injections of the drug.

*Treatment with Multiple Doses of Ethylhydrocupreine Injected into Both Pleural Sacs.*—Additional experiments were conducted in which daily injections of 0.5 cc. of culture were given into the right pleural sac of guinea pigs. Treatment began 4 hours after the second injection of culture and was repeated daily 4 hours after each injection of culture; it consisted in the injection of 0.5 cc. of 1:500 ethylhydrocupreine into each pleural sac. The object of these experiments was to produce a milder pleuritis permitting longer duration of life. The fact that the first treatment was delayed 24 hours allowed the development of acute pleuritis which was generally confined to the right side. The conditions occurring in pneumococcus pleuritis in man were more nearly approached by administering the drug 4 hours after the culture.

The results of one experiment are shown in Table VII. Untreated controls usually died within 4 or 5 days, while controls that received drug alone survived after multiple injections. The results were usually irregular but practically always showed a distinct curative influence of the drug. Animals either survived for several days after the death of untreated controls or lived until killed for the purpose of making gross and histological studies of the pleuræ.

TABLE VII.

*Effect of Ethylhydrocupreine upon Pleuritis in Guinea Pigs.*

0.5 cc. of culture injected into right pleural sac. 24 hours later 0.5 cc. of culture injected into right pleural sac and daily thereafter. 4 hours after second injection of cocci, 0.5 cc. of 1:500 ethylhydrocupreine injected into both sacs, a dose equivalent to about 2.5 cc. per kilo. Injections repeated 4 hours after each injection of cocci.

Weight of guinea pig.	No. of injections of culture.	No. of injections of drug.	Result.
<i>gm.</i>			
500	4	3	Lived.
460	4	3	Died 7 days after first dose of culture;* small area of plastic pleuritis on right side; no changes in left pleura.
470	4	None (control).	Died 4 days after first dose of culture;† very extensive bilateral pleuritis and pericarditis.
400	None (control).	3	Lived.

\* No pneumococci in heart's blood or exudate.

† Pneumococci in heart's blood and exudates.

Similar results were observed with dogs weighing about 5 kilos. These animals were infected by injecting into the right pleural cavity 15 cc. of a 24 hour broth culture reinforced by the cocci removed from ten blood agar slant cultures. Treatment was begun 24 hours after infection by injecting 15 cc. of a warm 1:500 solution of ethylhydrocupreine into the right pleural cavity and was repeated daily until four to seven doses had been given.

In an experiment of this kind the untreated control animal died 72 hours after infection with pleuritis of the right side and acute hyperemia and serous exudation of the right lung (Fig. 11); pneumococci

were recovered by culture from the heart's blood and pleural exudate. The left lung and pleura were not involved.

The treated animal received six injections of ethylhydrocupreine and survived. 24 hours after infection and just before the first treatment was begun, the animal appeared sick and the leucocytes had increased from 11,600 to 17,400 per c.mm. of blood. 24 hours after the first treatment the leucocytes were 17,000 per c.mm. and gradually fell each day until 9,400 were reached on the 6th day. On this day the rectal temperature was 38°C. and treatment ceased. This animal was in normal clinical condition when necropsied; the lungs and pleura on both sides were normal, there were no exudates, and cultures of the heart's blood and contents of the pleural sacs were sterile.

*Treatment with Single Doses of Ethylhydrocupreine, Sodium Oleate, and Boric Acid Injected into Both Pleural Sacs.*—Since Lamar (6) found that in the presence of boric acid, which overcomes the inhibitory influence of proteins, sodium oleate in 0.5 to 1 per cent solution is

TABLE VIII.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 0.5 Cc. of a Mixture Composed of Equal Parts of 1:100 Ethylhydrocupreine, 0.5 Per Cent Sodium Oleate, and 5 Per Cent Boric Acid into Each Pleural Sac at Varying Intervals after Infection.\**

Interval between time of infection and injection of mixture.	Result.
<i>hrs.</i>	
Immediately.	Lived.
2	"
4	"
24	"
24	"
Culture alone (control).	" about 48 hrs.;† severe bilateral suppurative pleuritis and pericarditis.
Mixture alone (control).	Lived.

\* Animals infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

† Pneumococci in heart's blood and exudates.



destructive for pneumococci and increases their susceptibility to the destructive influence of antipneumococcus serum, we have experimented with a mixture prepared of equal parts of 1:100 ethylhydrocupreine, 0.5 per cent sodium oleate, and 5 per cent boric acid. 0.5 cc. of this mixture was injected into each pleural sac; 1 cc. or more of the mixture injected into each sac proved toxic.

The results of an experiment are shown in Table VIII. Mixtures of sodium oleate and boric acid alone had little or no influence, but with the addition of ethylhydrocupreine the results were frequently very successful and decisive, animals surviving even when infected 24 hours before treatment was begun.

*Treatment with Single Doses of Ox Bile Injected into Both Pleural Sacs.*—Since ox bile causes lysis and destruction of pneumococci, experiments were undertaken<sup>3</sup> with injections of fresh sterile bile into both pleural sacs of guinea pigs at varying intervals after infection. Preliminary experiments showed that injections of 0.5 to 2 cc. of undiluted bile into each pleural cavity of guinea pigs weighing from 350

TABLE IX.

*Effect upon Pneumococcus Pleuritis in Guinea Pigs of Injecting 0.2 Cc. of Sterile Ox Bile\* into Each Pleural Sac at Varying Intervals after Infection.†*

Interval between time of infection and injection of bile.	Result.
hrs.	
2	Lived 48 hrs.;‡ bilateral pleuritis with extensive serous effusion.
4	" 48 " † " " " " " "
24	"
24	" 4 days; no exudate; adhesions on right side.
Culture alone (control).	" 48 hrs.;‡ severe bilateral suppurative pleuritis and pericarditis.
Bile alone (con- trol).	"

\* This is a dose equivalent to about 0.5 cc. per kilo.

† Animals were infected by injecting 1 cc. of a 24 hour broth culture into the right pleural sac.

‡ Pneumococci in heart's blood and serous exudates.

<sup>3</sup> These experiments were undertaken at the suggestion of Dr. Allen J. Smith.

to 400 gm. were toxic, the animals dying at once or surviving less than 24 hours. In these experiments undiluted bile in a dose of 0.2 cc. was injected into each pleural cavity; the controls survived this amount and showed no toxic effects.

The results of one experiment are shown in Table IX. Occasionally a treated animal survived longer than the untreated controls. Even when treated animals succumbed, the macroscopic lesions appeared less extensive and the exudates more serous and less purulent in character.

The experiments were interrupted at this point before treatment with mixtures of bile and ethylhydrocupreine could be tried. Bile alone appeared to exert some beneficial influence but our experiments in this direction have not been sufficiently numerous to warrant further statements.

#### DISCUSSION.

##### *Chemotherapy in Infections of the Serous Cavities.*

Undoubtedly ethylhydrocupreine hydrochloride has been proved by numerous investigators to show an extremely high and selective pneumococidal activity *in vitro*, even in the presence of proteins, as in menstria of serum and transudates. These investigations have also disclosed a certain degree of protective power of the base in oil when administered subcutaneously, or the soluble hydrochloride salt when administered intraperitoneally in mice infected with lethal amounts of pneumococcus culture.

Unfortunately, the drug has proved too toxic when given by mouth or by intramuscular injection in the treatment of lobar pneumonia in man, to permit the administration of sufficiently large amounts to influence materially the severity and mortality of the disease. The transient toxic effects observed have been such symptoms as tinnitus, deafness, amblyopia, or retinitis. The retinitis, or amaurosis, may, however, result in more or less permanent impairment of vision.

Moore and Chesney (7) have shown, however, that when 0.024 gm. of ethylhydrocupreine hydrochloride per kilo of body weight, or about 1.7 gm. per 70 kilos, are administered daily by mouth and when the intervals between doses are not longer than 3 hours, a specific pneumococidal property may be imparted to the blood which is maintained more or less constantly for several days.

In the treatment of pneumococcus meningitis and pleuritis it would appear that sufficient amounts of the drug may be injected intrathecally or intrapleurally to exert considerable pneumococcidal activity without irritating these membranes or causing toxic effects. When the drug is administered by mouth, intramuscularly, or intravenously, it is so highly diluted in the blood that enough cannot be administered to raise the pneumococcidal activity of the blood sufficiently for pronounced curative effects without danger of toxicity, but in the subarachnoid and pleural sacs dilution is by spinal fluid or exudate alone and is much less than in the blood.

The experiments by Kolmer and Idzumi in the treatment of experimental pneumococcus meningitis with ethylhydrocupreine showed that in mixtures of equal parts of purulent spinal fluid and 1:1,000 to 1:2,000 solutions of ethylhydrocupreine complete destruction of very large numbers of virulent pneumococci occurred within 1 hour at 37°C. The intrathecal injection of 0.5 cc. of 1:500 and 1:1,000 solutions per kilo of body weight, when administered not later than 4 to 6 hours after the injection of culture, had a distinct beneficial effect on the course of experimental meningitis in rabbits produced by Type I pneumococci. The drug was probably diluted in the subarachnoid space by 1 or 2 cc. of spinal fluid per kilo, giving final dilutions of 1:2,000 to 1:5,000; these dilutions of drug, however, apparently showed some pneumococcidal activity even in the presence of inflammatory exudates, and were found to have no irritative effects for the meninges. Furthermore, since the amount of drug injected at one time was 0.5 cc. of a 1:500 dilution per kilo of weight, or 0.001 gm., it was less than the toxic dose by intrathecal injection; this permitted the administration of multiple doses without toxic effects.

Similar but more decisive results have been observed in the present experiments. Since pleural pus is ordinarily much richer in pus cells it has required stronger solutions of ethylhydrocupreine to effect complete destruction of large numbers of pneumococci; in our experiments equal parts of pus and 1:1,000 solutions of ethylhydrocupreine kept at 37°C. are not completely sterilized in less than  $\frac{1}{2}$  to 1 hour.

Guinea pigs and dogs, however, show no ill effects from the intrapleural injection of 1 cc. of 1:500 ethylhydrocupreine per kilo. The toxic dose for guinea pigs appears to be approximately 2 cc. of a 1:100 solution per kilo, or 0.02 gm. of drug, so that the doses ordinarily employed in our experiments (1 cc. of a 1:500 solution for guinea pigs 350 to 500 gm. in weight in one or both pleural sacs) were at least two and one-half to five times less than the toxic doses.

The experimental lesions in guinea pigs were very severe, consisting of acute suppurative pleuritis which involved both pleuræ and the external portions of the pericardium. The intrapleural administration of ethylhydrocupreine in a dose corresponding to about 2 cc. of a 1:500 dilution per kilo of weight in each cavity, a total of 0.008 gm. of drug, had a marked curative influence in the majority of instances unless treatment was delayed longer than 24 hours. Such a delay permitted the development of severe bilateral pleuritis, pericarditis, and pneumococcus bacteremia. Multiple injections of 1 cc. of a 1:500 solution per kilo into each pleural cavity had an even more pronounced effect upon less severe infections.

These results indicate the possibility of chemical sterilization of infected serous cavities with drugs showing a marked and selective bactericidal activity for the infecting bacterium in the presence of pus, as optochin or ethylhydrocupreine hydrochloride for the pneumococcus, even though sufficient amounts of the drug cannot be administered by mouth, intramuscularly, or intravenously to influence a localized inflammatory process. It is highly probable that some of the drug injected into the cavity is absorbed into the blood, where it exerts some restraining influence upon the multiplication of bacteria.

#### SUMMARY.

1. Solutions of ethylhydrocupreine (optochin) hydrochloride show a pronounced bactericidal activity for pneumococci in pleural pus.

2. The highest tolerated dose of ethylhydrocupreine hydrochloride for guinea pigs by intrapleural injection is about 0.2 to 0.3 cc. of a 1:100 solution per 100 gm. of weight, corresponding to 2 to 3 cc. of solution or 0.02 to 0.03 gm. of the drug per kilo of weight.

3. The injection of 1 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride into each pleural cavity of a guinea pig weighing from 350 to 500 gm. produces no evidences of toxicity or irritation of the pleura.

4. The injection of 1 cc. of a 24 hour dextrose blood broth culture of virulent Type I pneumococci into the right pleural cavity of guinea pigs produces acute suppurative pleuritis on both sides associated with suppurative pericarditis, which generally terminates fatally within 72 hours with pneumococcus bacteremia.

5. The injection of 1 cc. of 1:500 solutions of ethylhydrocupreine hydrochloride into each pleural cavity of guinea pigs at varying intervals up to 24 hours after pleural infection has usually shown a marked curative influence. Similar results were observed with dogs.

6. The intrapleural injection of mixtures of ethylhydrocupreine, sodium oleate, and boric acid (Lamar) has also shown a decided curative effect in acute suppurative pneumococcus pleuritis of guinea pigs.

7. These and similar experiments on pneumococcus meningitis suggest that in chemotherapeutic investigations certain drugs may be injected into serous cavities in amounts exerting distinct bactericidal activity *in vivo* without producing local irritation or general toxic effects.

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## EXPLANATION OF PLATES.

## PLATE 87.

FIG. 1. Right lung and pleura of a guinea pig that received 1 cc. of 1:500 ethylhydrocupreine hydrochloride into each pleural cavity as a control on the animals shown in Figs. 12 and 13. This animal was living and well 7 days later when killed.

FIG. 2. Section of the right pleura and lung of a dog 4 days after the intrapleural injection of 15 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride per kilo of weight.

## PLATE 88.

FIG. 3. Acute bilateral suppurative pleuritis and pericarditis in a guinea pig 36 hours after the injection of pneumococcus culture into the right pleural cavity.

## PLATE 89.

FIG. 4. Film of pleural exudate showing large numbers of extracellular pneumococci 48 hours after the intrapleural injection of pneumococcus culture.

FIG. 5. Acute purulent pleuritis and pneumonia. Section of the right pleura and lung of a guinea pig 36 hours after the intrapleural injection of pneumococcus culture.

## PLATE 90.

FIG. 6. Acute purulent pleuritis of a guinea pig 48 hours after the intrapleural injection of pneumococcus culture. The exudate in the pleural space between the visceral and parietal layers is shown.

FIG. 7. Beginning organization of the exudate in acute pleuritis of a guinea pig 5 days after the intrapleural injection of pneumococcus culture.

## PLATE 91.

FIG. 8. Acute purulent pleuritis in a guinea pig 48 hours after the intrapleural injection of pneumococcus culture.

FIG. 9. Section of the right pleura and lung of a guinea pig that succumbed 48 hours after the intrapleural injection of pneumococcus culture.

## PLATE 92.

FIG. 10. Chronic fibrous (organized) pleuritis of a guinea pig following acute pneumococcus pleuritis.

FIG. 11. Edema, hyperemia, and leucocytic infiltration in the right lung of a dog 72 hours after the intrapleural injection of pneumococci; there was an accompanying acute fibrinous pleuritis.

## PLATE 93.

FIG. 12. Lung of the animal shown in Fig. 9.

FIG. 13. Section of the right pleura and lung of a guinea pig that was infected at the same time and in the same manner as the animal shown in Figs. 9 and 12, but received 1 cc. of 1:500 ethylhydrocupreine into each pleural cavity 4 hours after infection. This animal was living and well 7 days later when killed.

FIG. 14. Section of the right pleura and lung of a guinea pig that was infected at the same time and in the same manner as the animal shown in Fig. 8, but received 1 cc. of a 1:500 solution of ethylhydrocupreine in each pleural cavity 24 hours later. The animal was living and well 5 days later when killed.

## PLATE 94.

FIG. 15. Lungs, pleuræ, and pericardium of a guinea pig that was infected at the same time and in the same manner as the animal shown in Fig. 3 but received 1 cc. of a 1:500 solution of ethylhydrocupreine hydrochloride in each pleural cavity 4 hours after infection and two subsequent injections at daily intervals. This animal was living and well 5 days later when killed.





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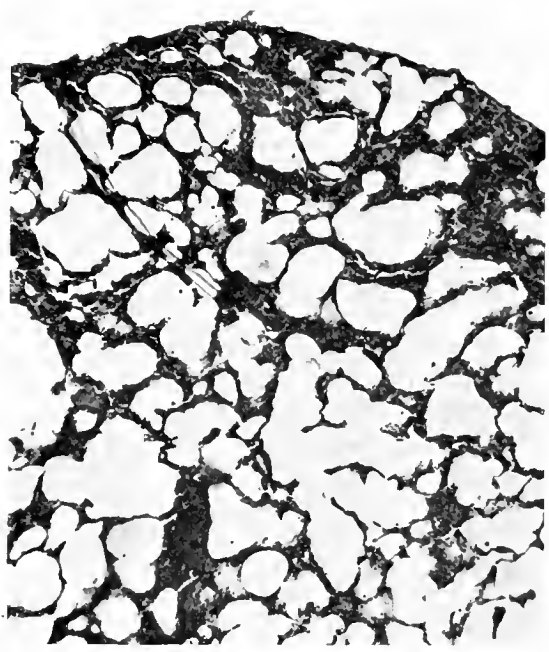


FIG. 1.

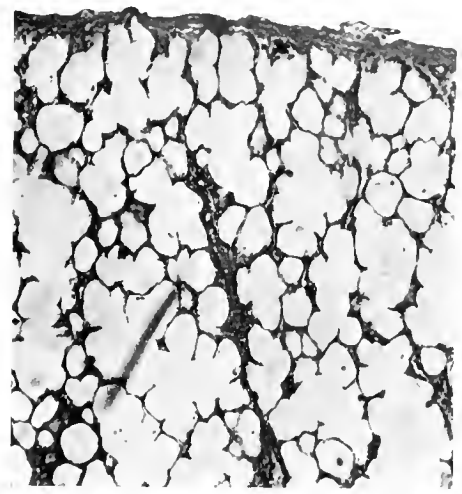


FIG. 2.

(Kolmer and Sand: Pneumococcus pleuritis.)





FIG. 3.

Kolmer and Sand : Encephalomyelitis.



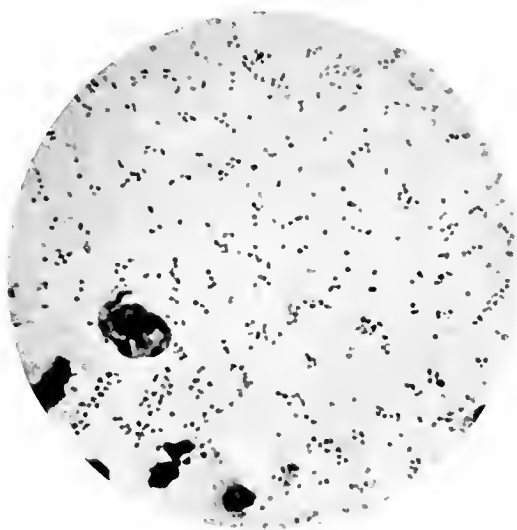


FIG. 4.

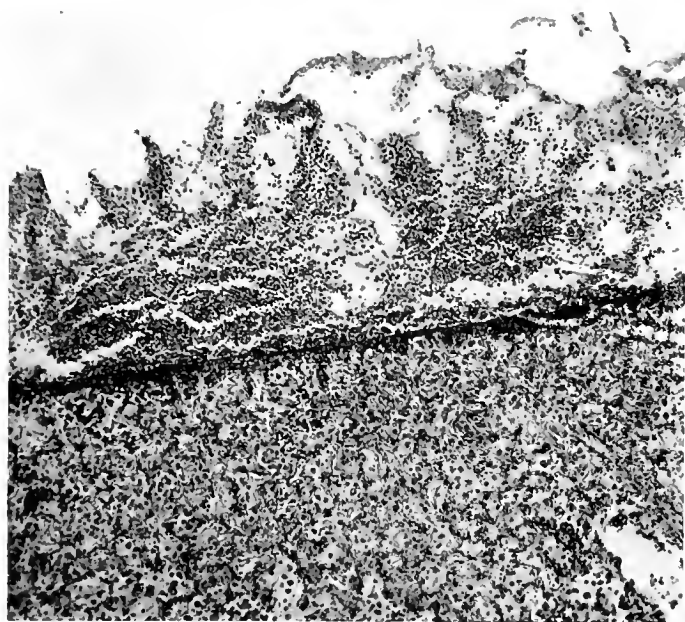
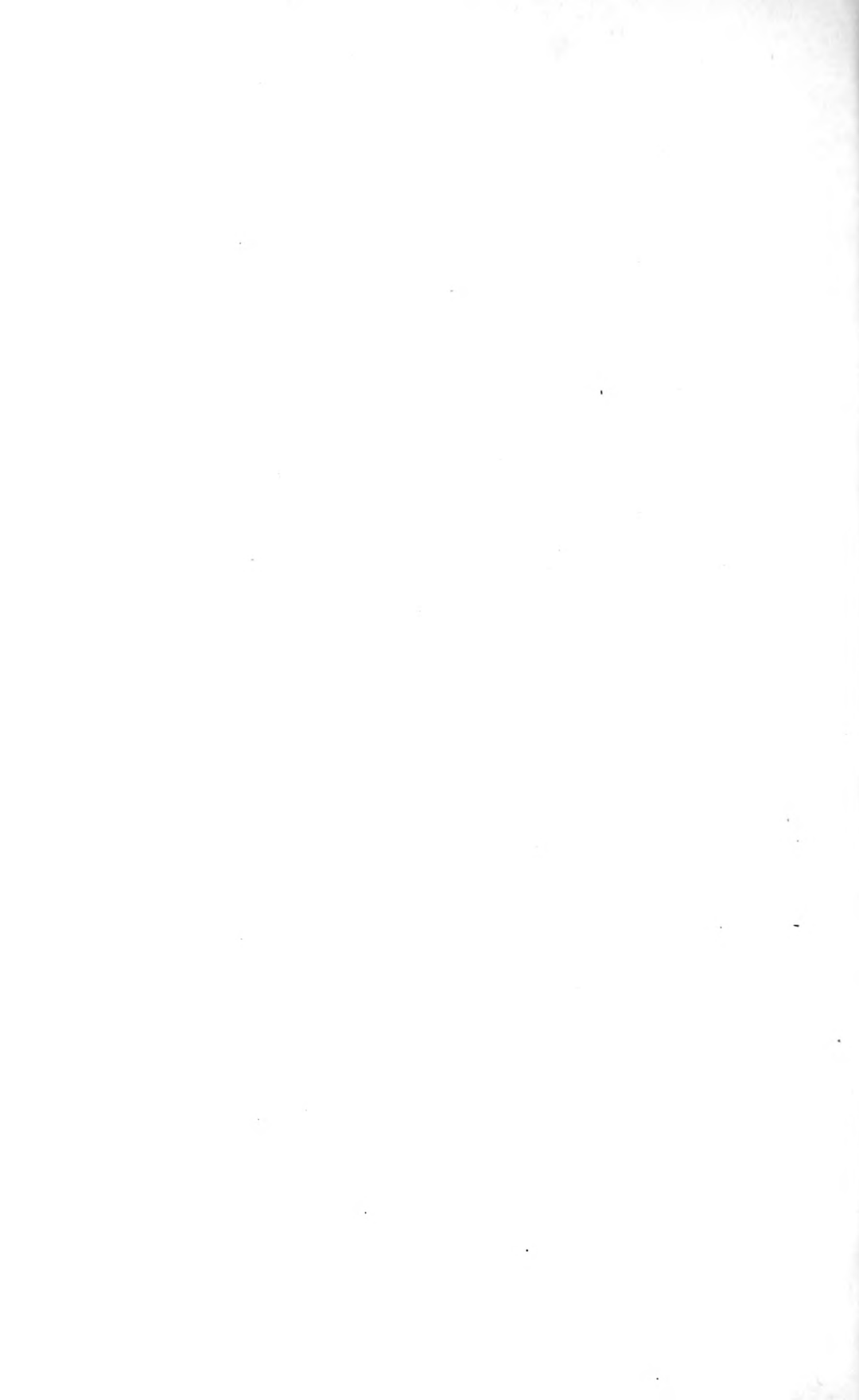


FIG. 5.

(Kolmer and Sands: Pneumococcus pleuritis.)



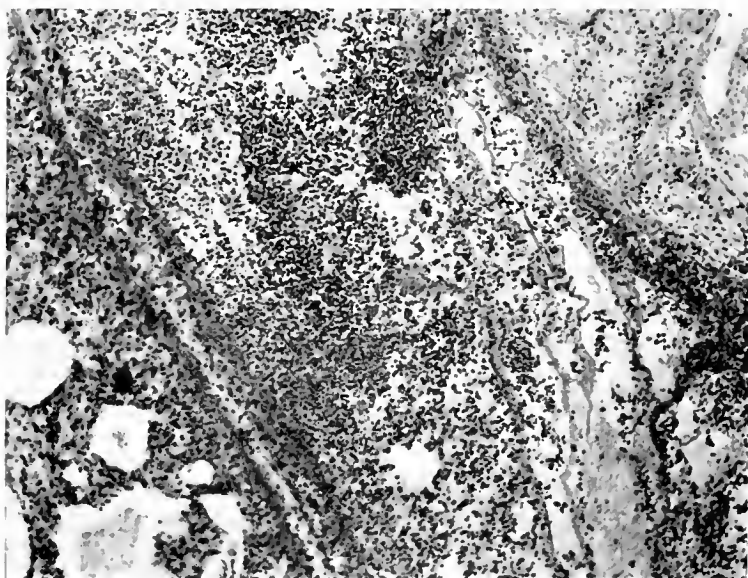


FIG. 6.



FIG. 7.

(Kolmer and Sands: *Pneumococcus pleuritis*.)





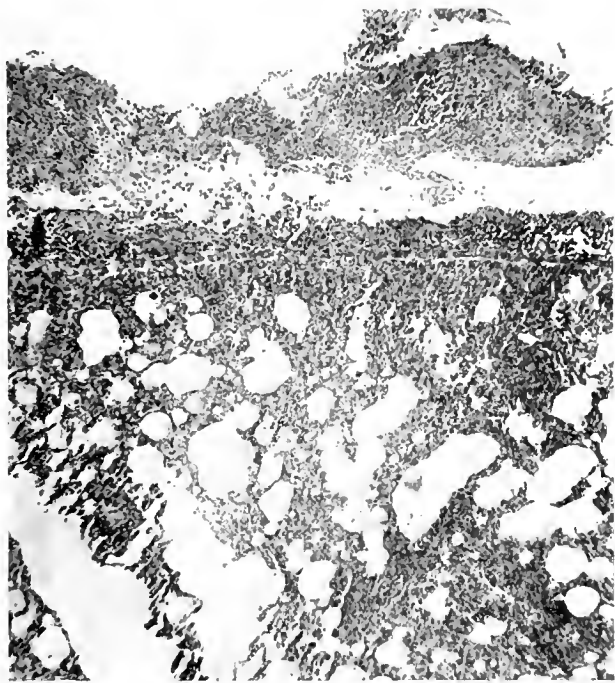


FIG. 8



FIG. 9.

(Kolmer and Sands: Pneumococcus pleuritis.)



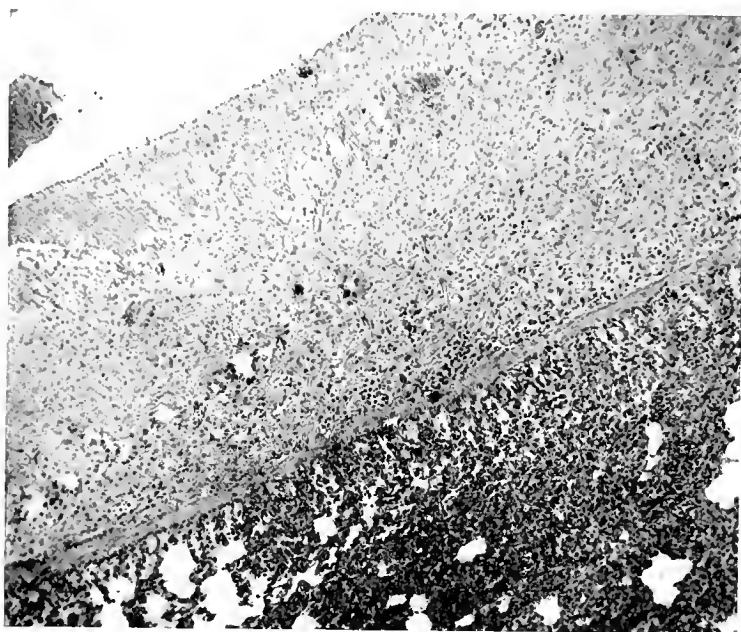


FIG. 10

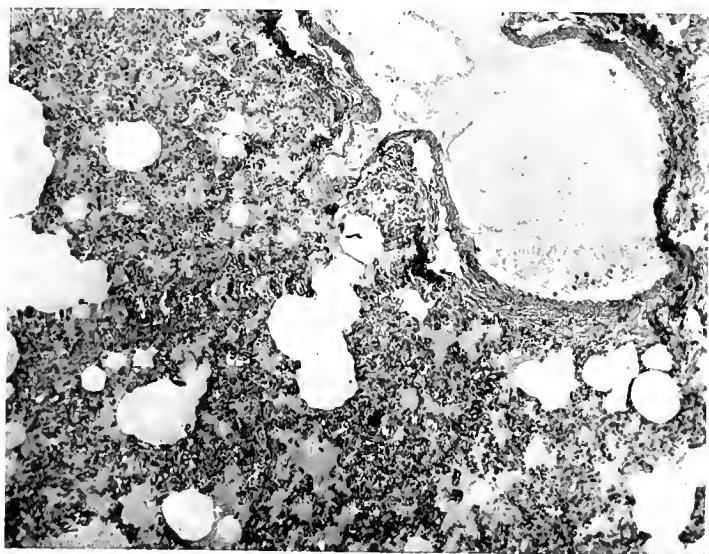


FIG. 11.

(Kolmer and Sand: Pneumococcus pleuritis.)



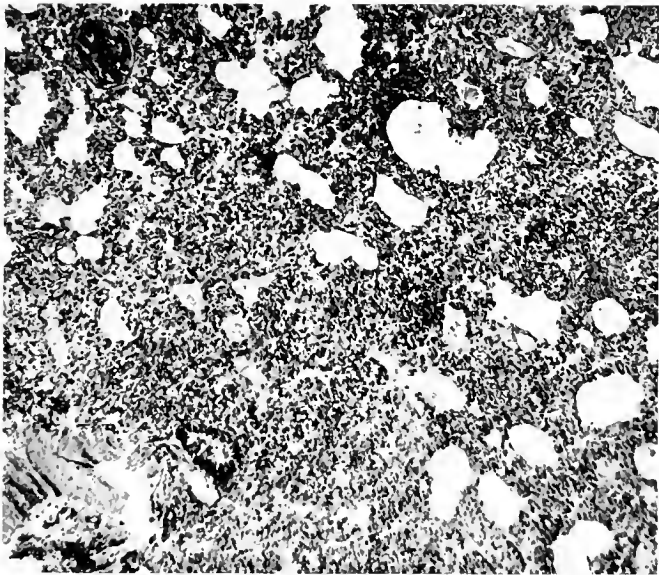


FIG. 12.

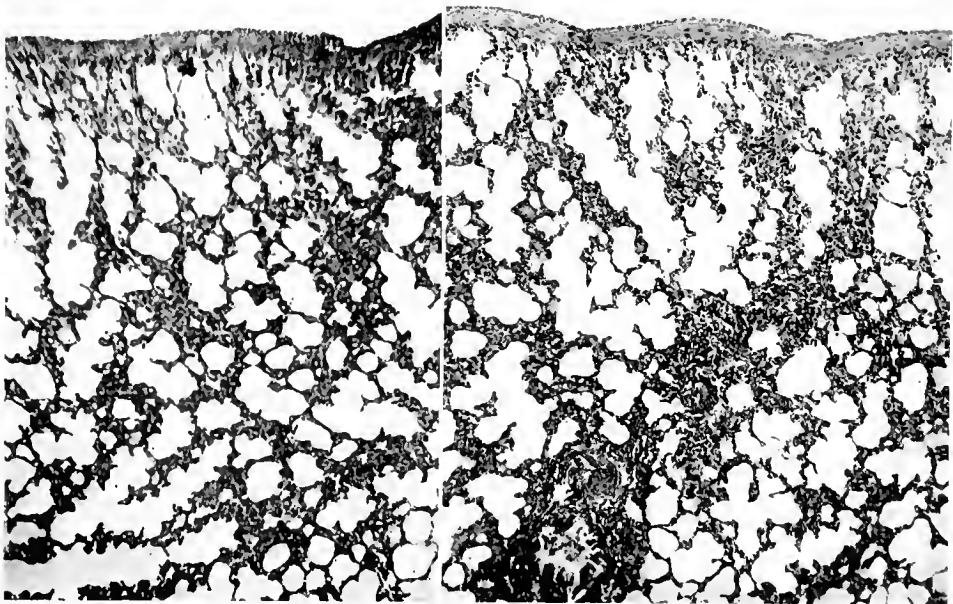


FIG. 13.

FIG. 14.

(Kolmer and Sands: *Pneumococcus pleuritis*.)





FIG. 15.

(Kolmer and Sands: Pneumococcus pleuritis.)





# EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

## IV. ANAEROBIC CULTIVATION.

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PLATES 95 TO 99.

(Received for publication, February 25, 1921.)

In a series of reports in this Journal<sup>1, 2, 3</sup> and in *The Journal of the American Medical Association*,<sup>4, 5</sup> we have described the changes in the blood and lungs of rabbits and guinea pigs which follow the intratracheal injection of unfiltered and filtered nasopharyngeal secretions, obtained within 36 hours after onset, from patients ill with uncomplicated epidemic influenza. The activity of the injected material was traced to the presence of a substance possessing characters which could only be attributed to a living agent, not, however, of the nature of ordinary bacteria. In the earlier reports<sup>1, 4</sup> we referred to experiments on the cultivation of this living agent by anaerobic methods, and recently<sup>5</sup> we described the nature of the characteristic, visible bodies usually found by cultural methods to be present in the nasopharyngeal secretions during the early hours of epidemic influenza in man and in the lung tissue of affected animals. The present paper describes these cultivation experiments in greater detail.

<sup>1</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>2</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

<sup>3</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

<sup>4</sup> Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1920, lxxiv, 1497.

<sup>5</sup> Olitsky, P. K., and Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvi, 640.

*Sources of Material.*

The characteristic bodies were first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Since that date successful cultivation experiments have been carried out with material ultimately derived from all of the seven cases of influenza from which active material was transmitted to animals. Numerous primary cultures, directly from human sources or from the lung tissues of affected animals, have been followed by hundreds of subplants, until at present certain strains are extant in the eighteenth generation.

The materials subjected to cultivation, essentially the same as those injected in the transmission experiments, were obtained from a variety of sources. During the two epidemic waves of influenza, that of 1918-19 and that of 1920, nasopharyngeal secretions were collected from patients with the uncomplicated disease, both in the early hours of the affection and in the later stages, including convalescence. During the first epidemic filtered and unfiltered material was also obtained from the lungs, at autopsy, of patients who had succumbed to secondary or concurrent pneumonias. In the course of the various series of transmission experiments with rabbits and guinea pigs, the lungs of affected animals were cultured both aerobically and anaerobically as a routine. Usually this material was not filtered. Sometimes the tissues were ground in saline solution and filtered in order to remove bacteria of the ordinary species. As a routine also, portions of the lungs of affected animals were preserved in sterile 50 per cent glycerol. These glycerolated specimens, after preservation for periods up to 14 months, were directly cultured or injected intratracheally into rabbits, from whose lungs cultures were made at autopsy.

The control materials for cultivation experiments were likewise similar to those used in control transmission experiments in animals. They consisted of unfiltered and filtered nasopharyngeal secretions from healthy persons during the two epidemic waves, the inter-epidemic recession, and the postepidemic period. Nasopharyngeal washings from persons suffering from acute coryza were also cultured

during the non-epidemic intervals. The normal lungs of uninoculated rabbits, the diseased lungs of stock rabbits who fell ill of other infections, the lungs of rabbits injected with control materials, and, finally, each of the various ingredients of the culture medium were subjected to anaerobic cultivation as controls.

### *Methods of Cultivation.*

All the methods of anaerobic cultivation employed involved the use of fresh sterile kidney tissue in tubes or flasks of human ascitic fluid or ascitic fluid agar, usually under a vaseline seal.

In the early cultivation tests sterile paraffin oil was used as a seal instead of vaseline, and the tubes were incubated in an anaerobic jar set up as described by McIntosh and Fildes.<sup>6</sup> The decolorization of a tube of methylene blue in broth, included in the jar, indicated the establishment of anaerobic conditions. In later experiments the complete exclusion of oxygen by a vaseline seal permitted the rapid production and the maintenance of anaerobic conditions by the reducing substances in the medium.<sup>7</sup> The use of the anaerobic jar was, therefore, discontinued.

For primary isolations and routine cultivations the culture tubes were set up as follows: Relatively large pieces (0.6 to 0.8 gm.) of sterile normal rabbit kidney tissue were placed in test-tubes measuring 20 by 1.5 cm., one piece to each tube. Then 1 cc. of suspected fluid or 0.5 cm. of affected lung tissue was placed directly on the kidney tissue. These materials were covered with 8 to 10 cc. of sterile human ascitic fluid of a hydrogen ion concentration of 7.8 to 8. Ascitic fluids of a higher alkalinity were discarded. Sealed with 2 cc. of melted sterile vaseline and stoppered with cotton, the tubes were incubated at 37°C. for 8 to 12 days.

For special purposes this routine culture method was modified in two particulars. Occasionally tubes of a semisolid medium or mass cultures in flasks were employed, especially for subplants. The semisolid medium consisted of 1 part of beef infusion (2 per cent) agar, pH 7.4, to 2 parts of human ascitic fluid, mixed in a flask at 40°C.

<sup>6</sup> McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

<sup>7</sup> Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

and pipetted onto the kidney tissue and inoculum in the tall test-tubes. The medium for mass cultures was slightly modified from that described by Flexner, Noguchi, and Amoss<sup>8</sup> for the cultivation of the globoid bodies of poliomyelitis. In each Florence flask, of 50 cc. capacity, was placed one-fourth of a moderate sized rabbit kidney. The sections were cut across the entire kidney and placed with the cut surface parallel to the base of the flask. After inoculation the kidney was just covered (10 cc.) with the semisolid ascitic fluid medium described above and the agar was hardened by immersion of the flask for 15 minutes in cold water. Then the flask was filled to the neck with a mixture of 1 part of beef infusion broth, pH 7.4, and 2 parts of ascitic fluid. A seal of vaseline, 1 cm. deep, and a cotton stopper completed the preparation.

An advantage of the vaseline seal over the anaerobic jar was found in the ease with which specimens for examination or transplant could be obtained with a capillary pipette without exposure of the medium to the air. After puncture the seal was restored by gentle heat—just sufficient to melt a portion of the superposed vaseline.

All the earlier inoculations were made directly on the kidney tissue before the medium was added. More recently the incubation period has been shortened and growth facilitated by inoculating sterile preparations set up a day or two in advance, to permit the establishment of anaerobic conditions under the vaseline seal. The inoculum is placed in the vicinity of the kidney by means of a capillary pipette.

In primary cultivations and early subplants the suspected material was distributed among three to six culture tubes. Frequently only a few tubes of several in an initial cultivation showed growth. But apparent failure in the initial cultivation might be followed by success in a subplant. Hence at least two negative cultivations in succession were required before a culture series was discarded as negative.

#### *Appearance of Positive Cultures.*

The first evidence of the multiplication of a living substance in primary cultures in fluid medium was observed in the presence of a faint haze, first visible about the 5th day, extending upward about

<sup>8</sup> Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

1 cm. from the level of the kidney. This faint even cloud gradually became denser, reaching its maximum about the 8th day, when it approximated 3 cm. in depth. At the same time the clear supernatant fluid developed a characteristic very faint opalescence, often hardly discernible. On standing at room temperature the cloud gradually settled down, in the course of 2 weeks, to the region of the kidney tissue, leaving a clear supernatant fluid.

Initial cultures in a semisolid medium usually failed to show signs of growth. Subplants from the fluid medium developed slowly in semisolid cultures with the formation of almost microscopic colonies, too small to be defined with exactness. The evidence of growth was an even clouding of the medium extending to 3 to 2 cm. from the surface.

In mass cultures of well established strains a turbidity appearing throughout the semisolid layer about the 3rd day was followed by a diffuse clouding of the fluid portion by the 5th day. On standing at room temperature the cloud gradually subsided to the vicinity of the agar, forming a dense homogeneous nebulous layer on its surface (Fig. 1).

#### *Morphology and Staining Reactions.*

The greatest concentration of cultivable bodies for microscopic examination was obtained from the very bottom of the culture tubes. 0.3 to 0.4 cc. of the cloudy sediment was drawn up in a capillary pipette. After removing the adherent vaseline from the pipette with gauze the first two drops of fluid were discarded and subsequent drops were spread evenly on a slide in thick films. The films were then dried in an incubator at 37°C., fixed with gentle heat—three rapid passages through the Bunsen flame—and stained by the chosen method.

Of all the stains tried, well ripened Loeffler's alkaline methylene blue proved the most satisfactory. The preparation was flooded with the dye and steamed very gently over a flame for 2 minutes. The slide was thoroughly washed with running tap water and allowed to dry in the air.

Prepared in this manner, the films from typical cultures revealed minute bodies of regular morphology, stained a deep purple, and

clearly differentiated from the background of pale blue protein precipitate. The bodies were often exceedingly numerous, and with careful focusing, they stood out in sharp relief. Usually solitary, they were often found in diplo forms, and occasionally in short chains of three or four members. Clumps occurred also, especially in older cultures, the discrete definitely stained bodies forming masses from a blood platelet to a leucocyte in size. Viewed with the highest powers of the microscope, the bodies were seen to be two to three times longer in one direction than in the other. They were, therefore, bacilloid rather than coccoid. Thus they were differentiated sharply from the globoid bodies of poliomyelitis, which they approached in size. Their long axis measured 0.15 to 0.3 microns. Occasionally longer individuals were seen, but the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape. Irregular staining reactions have not been encountered. No granules, clubs, spores, or involution forms have been seen. The bodies in one culture in its eighteenth generation are morphologically identical with those in the initial specimen (Figs. 2 to 5).

Occasionally unmistakable clumps or colonies developed in the region of the kidney (Fig. 5), but when few in number the bodies were sometimes obscured by protein or stain precipitates (Fig. 4). Under such circumstances, the unaccustomed eye made them out with difficulty. For this reason stains which showed any tendency to precipitation on the slide were found unsuitable for their demonstration. Thus, although the bodies were died lavender with Giemsa's, Wright's, and Manson's stains, the preparations were as a rule unsatisfactory. Carbolthionine (Nicolle), carbolfuchsin, steaming safranine, and steaming fuchsin were likewise unsuitable. All the strains examined decolorized uniformly by Gram's method. The counterstain, safranine, required steaming. The Gram-negative reaction was a constant feature of young and old cultures.

The dark-field microscope has not afforded a satisfactory method of examination on account of the similarity in size of the cultivable organism to the familiar dancing bodies of control preparations.

*Filterability.*

The cultivable bodies, even in remote generations, have been found to pass Berkefeld V and N candles. They are, therefore, to be classed among the filter passers.

*Cultural and Biological Characters.*

The strict anaerobic and nutritive requirements of the cultivable bodies have necessarily limited the study of their cultural reactions. Certain conclusions have been drawn from repeated experiments.

*Final Hydrogen Ion Concentration of Cultures.*—The incubation of sterile kidney tissue in ascitic fluid has been shown to change the hydrogen ion concentration of anaerobic tissue cultures toward the acid side.<sup>7</sup> In our experiments the final hydrogen ion concentration of growing cultures and of uninoculated controls was the same; namely, pH 7.4 to 7.8. The organisms failed to grow in media of a pH of 8 or 7, which appear to be the outside limits of the favorable range.

*Action on Carbohydrates and Alcohols.*—Growth took place in the presence of dextrose, maltose, lactose, saccharose, inulin, and mannitol. No observable amounts of acid or gas were produced. The media containing dextrose and maltose (1 per cent) showed a heavier cloud than usual, but this may or may not have corresponded to a more active multiplication of the organisms. No characteristic odor was detectable in any of the cultures.

*Symbiosis.*—In the course of the experiments certain culture tubes, inoculated with unfiltered lung tissue, yielded growths of ordinary bacteria in addition to the characteristic bodies seen in the original culture tubes and later demonstrated in pure culture with filtrates of the mixed material. It thus appears that this organism can develop in symbiosis with *Bacillus pfeifferi*, the pneumococcus, *Streptococcus hemolyticus*, *Streptococcus viridans*, and staphylococci. In a few experiments deliberate mixtures of these bacteria with the influenzal bodies were cultivated, and the organisms were subsequently separated by plating and by filtration.

*Resistance.*—No growth has been obtained in subplants of cultures heated to 56°C. for  $\frac{1}{2}$  hour or longer. Exposure to chloroform vapor for 1 to 1½ hours apparently destroys the organism. Viable organisms have been found, however, in fluid, semisolid, and mass cultures kept at room temperature, 20–24°C. (68–76°F.) for periods up to 6 months.

In these respects the resistance of the cultivated bodies is similar to that of the active agent in glycerolated specimens of the lung tissue from affected rabbits.<sup>2</sup>

*Enumeration of Positive Cultures and the Sources from Which They Were Obtained.*

In the earliest experiments, before a precise technique for culturing and demonstrating the cultivable bodies was developed, it may be presumed that some active materials gave negative results. Hence the following enumeration of successful cultivations is of more significance from the positive than from the negative point of view, and cannot be regarded as an indication of the actual incidence of these cultivable bodies in epidemic influenza in man. This is particularly true in view of the fact that cultural experiments were not accepted as positive unless at least two generations of the cultivable bodies were obtained.

Cultivation experiments were attempted with the filtered nasopharyngeal washings of eleven patients with uncomplicated epidemic influenza during the first 36 hours of the illness. Of these cultivations, six gave positive results. Five strains were obtained from eight patients in the 1918–19 epidemic and one strain from three patients in the recurrence of 1920.

Material from twenty-eight other patients was cultured during the later stages of the disease—from the 48th hour to convalescence. Only one culture yielded a growth. This material was obtained 48 hours after onset from a patient who died 2 days later from a secondary pneumonia.

The filtered nasopharyngeal washings of four patients suffering from pneumonias secondary to epidemic influenza were apparently negative. No growth was obtained with filtrates of the lung



tissue, at autopsy, of two patients who succumbed to the secondary pneumonias.

Although the cultivation of these peculiar anaerobic bodies from the lung tissues of a large number of affected rabbits and guinea pigs in the transmission experiments already described<sup>1, 2, 3</sup> is presumptive evidence that this organism was probably the causative agent in the lesions produced, it is nevertheless important to correlate the presence of these organisms in the human nasal washings with the pathogenicity of these washings for rabbits.

The transmission experiments were initiated with the nasopharyngeal washings of seven patients, all in the first 36 hours of illness. Three of these specimens, filtered and cultivated, yielded strains of the characteristic organism. The specimens from three other patients failed to produce a growth. The seventh specimen was not cultivated. An eighth specimen, obtained early, appeared to be negative both in cultivation and animal transmission experiments. Three strains of the cultivable bodies were obtained from specimens of nasopharyngeal washings which were not used for transmission experiments in rabbits.

Strains were ultimately derived, however, from all seven of the patients enumerated above by cultivation of the lung tissues of rabbits and guinea pigs affected in animal transmissions of the active nasopharyngeal material.

Beside the unfiltered and the filtered nasopharyngeal washings and the fresh lung tissues of affected rabbits and guinea pigs, a third source of active material, pathogenic for rabbits, was rabbit lung tissue which had been preserved in sterile 50 per cent glycerol for periods up to 9 months.<sup>2</sup>

None of the specimens of glycerolated lung which were directly cultivated yielded growths of the specific organism. We have already reported<sup>2</sup> the activity of certain specimens of the glycerolated material in initiating characteristic lesions when injected intratracheally in rabbits. From the fresh lung tissues of these affected animals, or their successors in the line of animal passage, the anaerobic bodies were cultivated in a number of instances. In this way the primary cultivation of rabbits' lungs from eleven series of experiments in which the active material had previously been immersed in glycerol

for 5 days to 9 months, yielded three cultures of these bodies; in one instance the length of glycerolation was 5 days, in another 1 month, and in the third 9 months. The two original sources of active material used in the above eleven series were Case 16,<sup>1</sup> representing the first epidemic period, and Case 26,<sup>1</sup> representing the second.

#### *Control Cultivation Experiments.*

Control cultivation experiments were made directly with the unfiltered or filtered nasopharyngeal secretions of twenty patients free from influenza. Eight of these patients were suffering from an acute coryza in the early or late stages. The control materials were collected in the epidemic, interepidemic, and postepidemic periods. None of these specimens yielded the cultivable bodies found in six of the eleven early cases of influenza examined.

Control cultures of the following materials also uniformly failed to yield growth of these bodies: the lung tissues of six stock rabbits which died of accidental or epidemic infections such as snuffles or pneumonia; uninoculated tubes of 36 samples of human ascitic fluid and portions of all the rabbit kidneys used in the culture media; and the lung tissues of 60 rabbits, either normal or injected intratracheally with control materials in the course of the transmission experiments. The control materials injected in these rabbits included normal rabbit lung tissue, saline solution, human ascitic fluid, rabbit serum, and ordinary bacteria.

#### *Inoculation of Rabbits and Guinea Pigs with the Cultivable Bodies.*

For a study of the effects of culture injections on animals, mass cultures<sup>9</sup> were generally used. Because of pressure of other experi-

<sup>9</sup> Mass cultures were prepared for inoculation as follows: The vaseline seal was removed from the Florence flask with a sterile wire and the fluid part of the culture centrifuged for 20 minutes at 1,500 revolutions per minute or until the supernatant fluid was clear. The fluid was then decanted, and the small button-like deposit of the growth left at the bottom of the tube was resuspended in saline solution. The centrifugation was repeated, the clear, supernatant saline solution was removed, and the small amount of sediment was again suspended in 4 cc. of saline solution, of which 3 cc. were used for the experiment.

ments most of the animal inoculations had to be deferred so that they were finally done with well established cultures several generations removed from the original human source.

When this part of the work was undertaken there were available for study three separate strains of cultivable bodies, two from the first epidemic and one from the second. These three strains were represented by cultures derived from nine different sources. One, a human strain, was the seventeenth generation subplant of a culture of the filtered nasopharyngeal secretion of Case 17<sup>1</sup> of the first epidemic. The others were first to seventh generation cultures of the lungs of different rabbits which had been injected with glycerolated lung tissue from earlier animals in the transmission series. Four of these cultures were thus originally derived from Case 16 and three were from Case 17 of the first epidemic. One came originally from Case 26 of the second epidemic of 1920.

Of these nine cultures with which rabbit passages were again initiated, only one, derived from rabbit lung tissue, and then in the third generation, failed to produce the effects regarded as typical for the active material in the earlier transmission experiments. The results of the intratracheal injection of the growth of mass cultures, in doses of 3 cc. were so uniform and familiar that a common description will suffice for the entire series.

On examination 24 hours after inoculation, the rabbits showed a rise in temperature and usually a conjunctivitis, varying from simple injection of the palpebral conjunctiva to a marked injection of the palpebral and ocular conjunctivæ. These signs were accompanied by a definite and often marked leucopenia, the result of a depression of the mononuclear cells (Text-figs. 1 and 2). In the animals which were kept for observation, these conditions persisted for 2 to 3 days, when the animals returned to normal. When the rabbits were killed during the reaction, a characteristic pathological picture was revealed in the respiratory organs.

Only the respiratory organs were visibly affected. The lungs (Figs. 6 and 7) were voluminous with edema and emphysema. Numerous hemorrhages were to be seen on the surface, either diffuse or discrete, and often in the form of minute petechiæ. The pleuræ were not

involved. On section of the lungs, the cut surface dripped a frothy blood-stained fluid, evidence of a hemorrhagic edema. Hemorrhages similar to those which had reached the surface were scattered through the parenchyma. The trachea and bronchi showed a mucopurulent exudate covering an exfoliated and hemorrhagic epithelium.

Microscopic sections (Figs. 8 and 9) confirmed the gross observations. The hemorrhages, diffuse or discrete, were located in the interalveolar tissue, which was distended with edema and torn by emphysema. The interalveolar structures were also infiltrated to some degree with a cellular exudate consisting of mononuclear cells and some polymorphonuclear cells with large eosinophilic granules. Large cells of the respiratory epithelial type, probably desquamated bronchial epithelium, and numerous erythrocytes were seen in the parenchyma. No pneumonic consolidation was present.

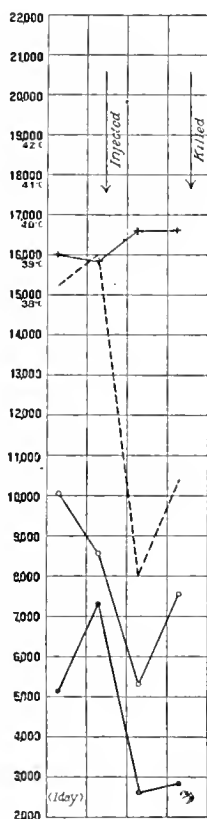
The bronchi showed thickened, hyperemic walls and their lumina were partly filled with erythrocytes, leucocytes, and fragments of exfoliated and necrotic epithelium. The lung capillaries were distended with blood.

As noted above, eight series of animal transmission experiments were initiated by the intratracheal injection in rabbits of pure cultures of the anaerobic bodies. From the lung tissues of rabbits in seven of these series the anaerobic bodies were recovered in pure culture.

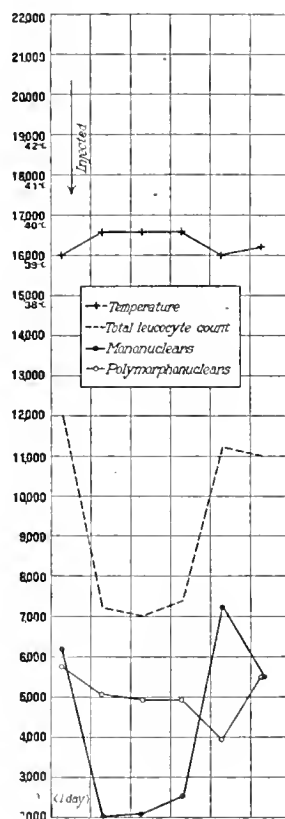
Both the strain obtained directly from the filtered nasal washings of Case 17, then in its seventeenth generation, and the strains derived from Cases 16, 17, and 26<sup>1</sup> after rabbit passage and glycerolation, produced the typical effects described above. The following protocols illustrate the similarity of the clinical effects produced by the human and the rabbit strains.

*Protocol 1.*—Preliminary observations on a rabbit for 2 days prior to inoculation gave the following results: temperature 39° and 38.9°C.; total leucocytes 15,200 and 16,000; mononuclear cells 5,168 and 7,360. Oct. 18, 1920. Inoculated intratracheally with 3 cc. of the growth of a second generation mass culture from the fifth rabbit passage of material from Case 16. Oct. 19. Temperature 39.6°C.; total leucocytes 8,000, of which 2,640 were mononuclears. Oct. 20. Temperature 39.6°C.; total leucocytes 10,400, of which 2,808 were mononuclears (Text-fig. 1). Rabbit killed. The lungs showed the hemorrhagic, edematous changes regarded as typical.

Another rabbit injected with the third generation mass culture of the same strain showed a similar picture of leucopenia and mononuclear depression. The rabbit was allowed to recover. This condition lasted for 3 days (Text-fig. 2).



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Effect on blood and temperature of the intratracheal injection of the cultivable bodies in a rabbit. The rise in temperature and the leucopenia due to mononuclear depression are noteworthy.

TEXT-FIG. 2. The same as Text-fig. 1 except that in this case the animal was allowed to recover. The persistence of the effects for 3 days is seen.

The next protocol is presented to show the effect of inoculation of a strain obtained by cultivating the nasopharyngeal secretions from a human case.

*Protocol 2.*—Jan. 6, 1921. A rabbit with normal temperature, 39°C., total leucocytes 10,400, of which 4,472 were mononuclears, was injected intratracheally with 3 cc. of the growth in the seventeenth generation mass culture of a strain from the filtered nasopharyngeal washings of Case 17. 24 hours later the animal showed a mild conjunctivitis, temperature 39.5°, total leucocytes 6,400, of which 1,152 were mononuclears. Jan. 8. The conjunctivitis was severe, total leucocytes 8,000, of which 2,400 were mononuclears. The lungs showed the lesions regarded as typical. The anaerobic bodies were recovered in pure culture from the lung tissue. The injection of this culture produced similar effects in a second rabbit, from the lung tissues of which the strain was again recovered.<sup>10</sup>

Several series of experiments were made with guinea pigs instead of rabbits. In this species the response to the intratracheal inoculation of cultures was similar to that obtained in rabbits, and from the affected lungs of the guinea pigs, the cultivable bodies were recovered.

Among the specimens of affected lung tissue preserved in 50 per cent glycerol were several which had come from rabbits injected with cultures of the anaerobic bodies. Although subsequent direct cultivations of these preserved specimens gave negative results, the same bodies were recovered from rabbit passages of the glycerolated material containing them. We thus have evidence that the bodies themselves do withstand glycerolation. The lungs from which these bodies were recovered by animal passage were immersed in glycerol for periods up to 4 months.

#### DISCUSSION.

These experiments seem definitely to connect the cultivable bodies with the clinical effects and lesions induced in rabbits and guinea pigs by the intratracheal injections of nasopharyngeal washings from patients with uncomplicated epidemic influenza.

From the lung tissues of such affected animals the morphologically and culturally characteristic bodies have been obtained in pure culture on special media by a strictly anaerobic technique. The bodies have been cultivated in successive generations without change in character. When injected intratracheally into rabbits and guinea

<sup>10</sup> The relation of ordinary bacteria in regard to their ability to produce concurrent or secondary infections in the presence of the cultivable bodies will form the basis of another paper.

pigs, they have given rise to pathological lesions in all respects similar to those from which they were obtained.

From the lesions the typical bodies have again been recovered in pure culture by the method employed for their primary isolation. Comparison of the strains thus derived from animal passages with those obtained directly from filtered human nasopharyngeal washings shows them to be identical in morphology and cultural characters. Finally, both the active material of the transmission experiments and the cultivable bodies obtained from similar sources withstand glycerolization and pass through Berkefeld V and N filters.

We feel, therefore, that the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism described in this paper.

It would, of course, be a simple matter to announce the inciting or etiological agent of epidemic influenza in man to be the minute, bacilloid organism here described. At present such a course does not seem desirable even though the clinical and pathological effects induced in the rabbit simulate so closely the phenomena found in epidemic influenza in man. Apparently we are at the threshold of our knowledge of a group or class of minute microorganisms which the anaerobic Smith-Noguchi technique has thrown open to exploitation. It seems wiser, therefore, to defer decision of the precise relation which the species described in this and previous communications bears to epidemic influenza until further experience is obtained.

In the meantime it is desirable to give the microorganism a name, and since a striking feature of its effect in rabbits is to diminish the resistance of the lungs to the action of ordinary pathogenic bacteria, as will be shown in a forthcoming paper, the name of *Bacterium pneumosintes* is proposed (from πνεύμων, lung, + σίντης, injurer, or devastator).

#### SUMMARY AND CONCLUSIONS.

From the filtered nasopharyngeal washings of patients in the first 36 hours of uncomplicated epidemic influenza and rarely in later stages of the disease, we have cultivated a minute bacilloid body,

*Bacterium pneumosintes*, 0.15 to 0.3 microns in length, of constant cultural characters and capable of indefinite propagation on artificial media. This organism, not of the nature of ordinary bacteria, was also recovered in pure culture from the unfiltered and filtered lung tissue of rabbits and guinea pigs inoculated with unfiltered and filtered nasopharyngeal washings of early influenza cases, both from the first epidemic of 1918-19 and from the second one of 1920. The organism grows only under strictly anaerobic conditions, passes Berkefeld V and N filters, and withstands the action of sterile 50 per cent glycerol for a period of months.

It has been recovered from cultures contaminated with a variety of ordinary bacteria such as *Bacillus pfeifferi*, pneumococci, streptococci, and staphylococci, and has been experimentally cultivated in symbiosis with them.

Similar cultivation of control materials uniformly failed to yield growths of this organism. The materials tested consisted of the unfiltered and filtered nasopharyngeal washings of persons free from influenza, some of whom were suffering from acute coryza, the lung tissue of normal rabbits and of rabbits with bacterial respiratory infections, and the uninoculated media.

The intratracheal injection in rabbits and guinea pigs of mass cultures of this organism has induced effects on the blood and lungs of these animals which are not to be distinguished from those obtained with the nasopharyngeal secretions of patients in the early hours of epidemic influenza. From the pulmonary lesions thus induced the same organism has been recovered in pure culture, and has been found to cause similar lesions on subsequent animal passage. Its pathogenicity is not lost by prolonged artificial cultivation.

Our experiments indicate that the cultivable bodies obtained directly from human nasopharyngeal washings and from affected rabbit lungs are strains of the same organism. This organism appears to be the source of the reactions which occur in experimental animals—rabbits and guinea pigs—as a result of the intratracheal injection of nasopharyngeal washings obtained during the early hours of uncomplicated epidemic influenza in man.



## EXPLANATION OF PLATES.

## PLATE 95.

FIG. 1. The construction of mass culture medium, to the left, and the growth of the cultivable bodies therein, to the right. The growth is derived from a rabbit's lungs after 8 days incubation. The turbidity of the semisolid and the haziness of the fluid layers are noteworthy. Actual size.

## PLATE 96.

FIG. 2. The cultivable bodies in the eighth generation. Culture obtained from a rabbit's lung into which was injected material, after 9 months immersion in glycerol, originally derived from the filtered nasopharyngeal secretions from a case of epidemic influenza. Stained with ripened Loeffler's alkaline methylene blue.  $\times 1,000$ .

FIG. 3. The bodies in Fig. 2 highly magnified. Stained similarly.  $\times 3,000$ .

FIG. 4. Comparative size of the cultivable bodies. An aerobic streptococcus and a chain of poliomyelitis globoid bodies, indicated by an arrow, have been superimposed. The cultivable bodies are very minute, uniform, and lightly stained, and are to be differentiated from the small irregular masses, deeply stained, which are protein precipitates. All  $\times 1,000$ .

FIG. 5. The cultivable bodies in colony formation.  $\times 1,000$ .

## PLATE 97.

FIG. 6. The gross lesions of the lungs of a rabbit inoculated intratracheally with the first generation of the cultivable bodies. The hemorrhages, edema, emphysema, and absence of pneumonic consolidation are noteworthy. Natural size.

FIG. 7. These lungs were obtained from a rabbit which was inoculated with the lungs pictured in Fig. 6. The more intense lesions of this second passage of the cultivable bodies are seen, especially the hemorrhagic edema of the right lung. Natural size.

## PLATE 98.

FIG. 8. Microscopic section of the lung lesions in a rabbit, caused by injecting the cultivable bodies intratracheally. The edema, the emphysema, the hemorrhages, and the cellular exudate are noteworthy.  $\times 240$ .

## PLATE 99.

FIG. 9. Another field of the same section shown in Fig. 8, demonstrating particularly the bronchial lesions. The necrotic and exfoliated bronchial epithelium and the edema of its walls are to be seen. The general edema and the vessel filled with blood may be observed.  $\times 240$ .





FIG. 1.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)





FIG. 2.

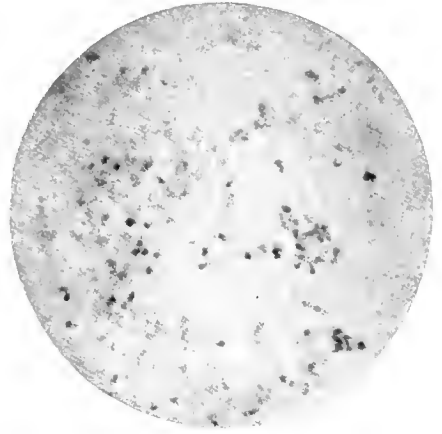


FIG. 3.

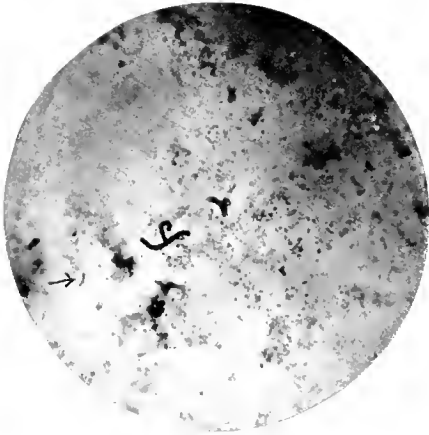


FIG. 4.



FIG. 5.





FIG. 6.



FIG. 7.





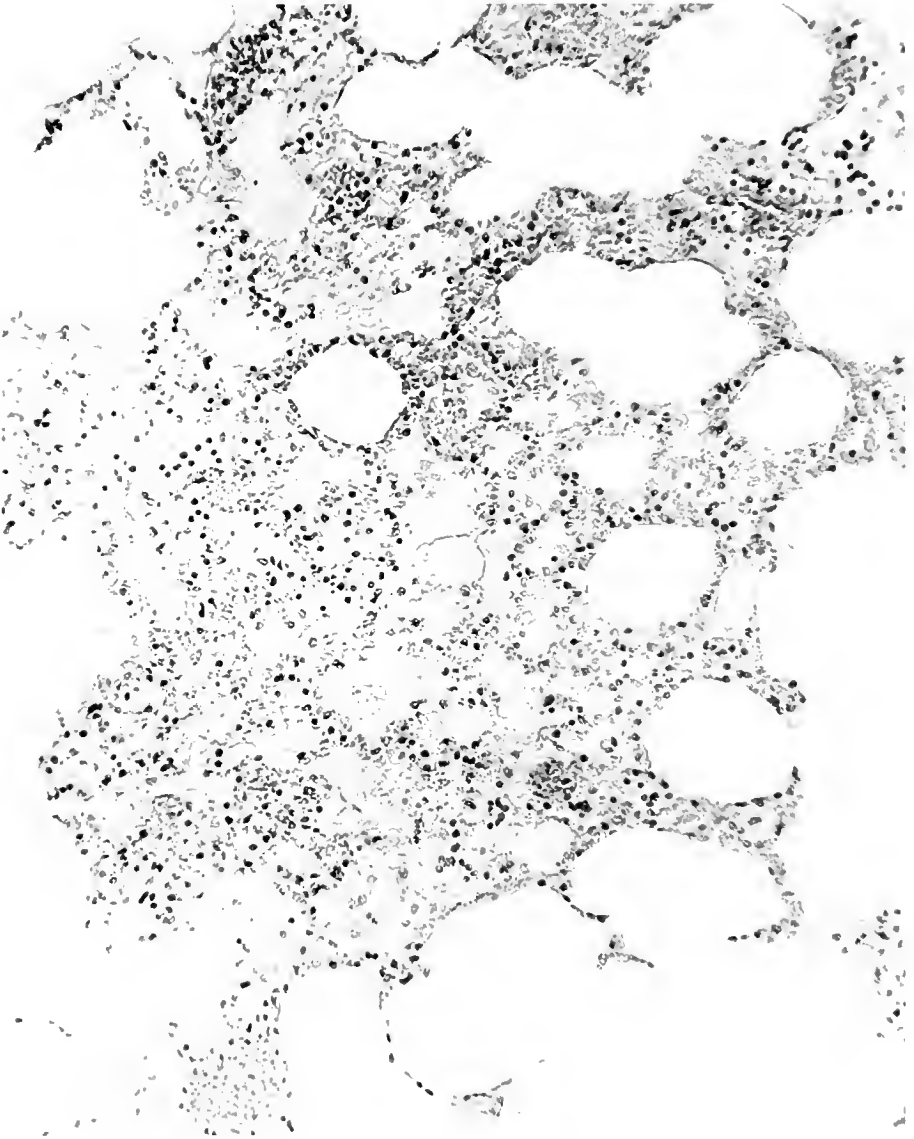


FIG. 8.

(Oltzky and Gate) — Nasopharyngeal secretions from guinea pig (IV.)



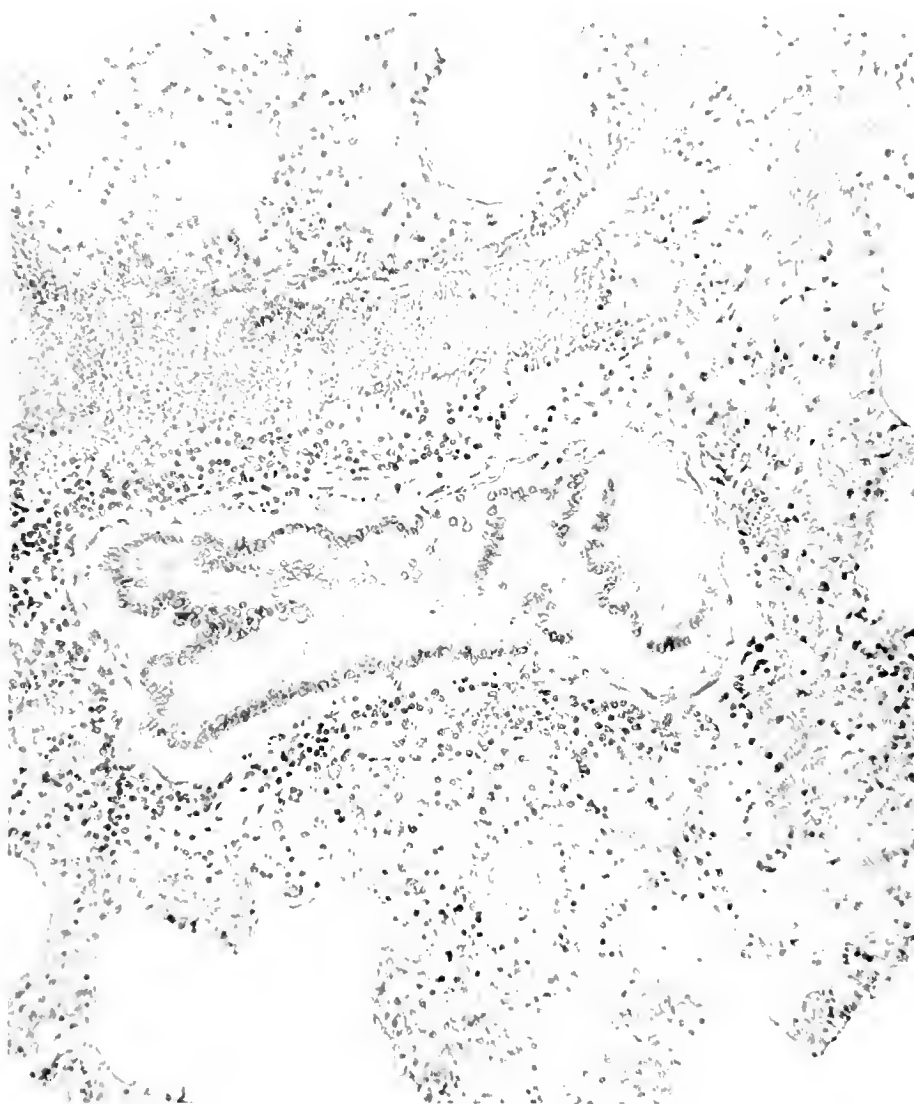


FIG. 9.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)



## THE BILIARY OBSTRUCTION REQUIRED TO PRODUCE JAUNDICE.

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(Received for publication, February 1, 1921.)

In a recent paper from this laboratory<sup>1</sup> observations were reported proving that a part of the rabbit's liver can function for the whole as regards bile elimination. A good instance in point is furnished by an experiment performed with another end in view. In a series of rabbits the ducts from three-fourths of the hepatic tissue were ligated and the portal branch to the remaining fourth was tied at the same operation, thus increasing the portal flow and by consequence bile formation in the mass with obstructed ducts. Under such circumstances the entire burden of bile elimination was laid on a mere quarter of the parenchyma, and this supplied with blood only by the hepatic artery, a source furnishing approximately two-fifths of the normal quantity if one may judge from data obtained in dogs.<sup>2</sup> Yet the rabbits remained unjaundiced and healthy. More recent observations upon dogs have convinced us that the experiment will yield a similar result with them. And it has been a surprise to discover on a search of the literature that no general recognition exists of the large margin of safety of the liver in bile elimination. On the contrary, frequent categorical statements may be found to the effect that, in man at least, jaundice is often caused by lesions affecting only a small proportion of the hepatic parenchyma.

Quincke and Hoppe-Seyler<sup>3</sup> state that transient obstruction of a small duct branch may bring about the absorption of sufficient bile to lead to a clinical

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<sup>1</sup> Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

<sup>2</sup> Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1914, xxxv, 87.

<sup>3</sup> Quincke, H. I., and Hoppe-Seyler, G., in Nothnagel, N., *Specielle Pathologie und Therapie*, Vienna and Leipsic, 2nd edition, 1912, xviii.

jaundice. The obstruction may be difficult to find at autopsy and the small ducts should be carefully searched for it. According to Eppinger<sup>4</sup> icterus is roughly proportional in intensity to the size of the occluded ducts, and localized inflammatory processes may cause it. Rolleston<sup>5</sup> believes that it follows the occlusion of one branch of the hepatic duct although the other continues to pour bile into the intestine; but not so according to Naunyn<sup>6</sup> who asserts that icterus under these circumstances fails to develop save when there is a complicating *Cholangie*. Krehl<sup>7</sup> holds that icterus occurs in many diseases through local stagnation and resorption. Recently van der Bergh and Snapper<sup>8</sup> have called attention to the fact that intrahepatic tumors of considerable size, and manifestly occluding many ducts, are often unaccompanied by jaundice, whence it follows in their opinion that local lesions in general must frequently exist without causing an accumulation of bile pigment in the organism.

The divergence of opinion illustrated by these specimen citations is obviously the result, first, of a lack of experimental evidence on the essential point at issue—the ability of a part of the liver to act for the whole as concerns bile elimination—and, second, of conclusions from clinical instances complicated by many factors.

For the work here to be described dogs and monkeys have been employed. Rabbits could not be used because their bile pigment fails to react satisfactorily to the ordinary tests.

#### *Criteria of Bile Retention.*

The clinician as a rule is first apprized of deficient bile elimination in his patient by the development of bilirubinuria with or without a tissue icterus. As our prime aim has been to determine how much biliary obstruction may exist without clinical manifestations, the tests in current use have been adopted for the work.

These tests as applied to the blood are very unsatisfactory. The Gmelin reaction with blood serum, so strongly advocated by French workers,<sup>9</sup> is yielded only when bilirubinemia is obvious to the eye, while furthermore lutein gives a

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<sup>4</sup> Eppinger, H., *Ergebn. inn. Med.*, 1908, i, 107.

<sup>5</sup> Rolleston, H. D., *Diseases of the liver*, London, 2nd edition, 1912.

<sup>6</sup> Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1919, xxxi, 537.

<sup>7</sup> Krehl, L., *Pathologische Physiologie*, Leipsic, 9th edition, 1918, 571.

<sup>8</sup> van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, i, 1109.

<sup>9</sup> Gilbert, A., Herscher, M., and Posternak, S., *Compt. rend. Soc. biol.*, 1903, lv, 530; 1905, lvii, 250.

positive reaction; and the intensity of color of the plasma itself, save in outspoken cases, is trustworthy only in the absence of hemolysis and of extraneous pigments such as carotin. The reactions of Obermeyer and Popper<sup>10</sup> have proved in our hands little more sensitive than that of Gmelin. The diazo test recently advocated by van der Bergh and Snapper<sup>11</sup> is delicate, and promises to be of great clinical value, but its use has been limited as yet and it is not entirely specific.<sup>12</sup> We have employed it in the present work in its negative aspect, as the criterion wherewith to rule out bilirubinemia, for which it would seem highly suitable. None of many dog sera obviously stained with bile has failed to give the test, and the unstained sera have regularly proved negative.

The diazo reagent is a mixture, made fresh each day, of the following stock solutions. (a) 5 gm. of sulfanilic acid and 50 cc. of hydrochloric acid in 1,000 cc. of distilled water. (b) 0.5 per cent solution of sodium nitrite in distilled water. For use 1 part of (b) is added to 50 of (a).

The oxalated or citrated plasma to be tested is first shaken briefly with two volumes of 95 to 96 per cent alcohol and centrifuged to throw out the precipitate that forms. The supernatant fluid will now contain all of the bilirubin unless the plasma held very large quantities, in which case some will be carried down with the precipitate and must be extracted with 64 per cent alcohol if a quantitative result is to be obtained. On the addition of the diazo reagent to the fluid containing bilirubin, in the proportion of 1 part to 4, the beautiful violet tint of azobilirubin appears after a few minutes. To determine its amount a colorimeter is used and a solution of pure bilirubin in chloroform (5 mg. per 100 cc.) to give with the reagent a standard tint.

The Gmelin reaction has been used to detect bilirubinuria, and to quantitate it Hooper and Whipple's<sup>13</sup> modification of Salkowski's method has been employed as routine. The sodium nitrite-nitric acid solution recommended by Gilbert, Herscher, and Posternak<sup>9</sup> has been found to yield a better Gmelin response than the ordinary fuming nitric acid.

In contrast with the tests just mentioned, those for bile salts in the urine are none of them satisfactory. Pettenkofer's reaction was employed for one series of dogs, but Hay's sulfur reaction was adopted for most of the animals as less open to technical error and relatively specific.<sup>14</sup> Attempts to detect cholates in the blood were after many trials abandoned. The method recently described by Hoover and Blankenhorn<sup>15</sup> whereby the salts are separated out by dialysis and

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<sup>10</sup> Obermeyer, F., and Popper, H., *Wien. med. Woch.*, 1910, lx, 2592.

<sup>11</sup> van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

<sup>12</sup> Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

<sup>13</sup> Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

<sup>14</sup> Lyon-Caen, L., *J. physiol. et path. gén.*, 1910, xii, 526.

<sup>15</sup> Hoover, C. F., and Blankenhorn, M. A., *Arch. Int. Med.*, 1916, xviii, 289.

TABLE I.  
*Bilirubinuria in the Absence of Bilirubinemia in Dogs.*

Normal dogs.				Operated dogs.				
Dog No.	Procedure.	Urine.	Blood.	Dog No.	Operation.	Time of observation.	Urine.	Blood.
16	Normal dog. Fasting on 6th and 9th to 12th days inclusive.	Bile pigment present on 6th and 8th to 14th days inclusive.	Negative.	19	All ducts ligated but those to lateral liver mass; 71 per cent of liver obstructed.	Before operation. After operation.	Bile pigment present for 3 days. Bile pigment present on 1st to 8th, and on the 10th day after operation.	Negative. Pigment in plasma on 6th, 7th, and 8th days after operation.
17	Normal dog. Fasting on 6th and 9th to 14th days inclusive.	Bile pigment present on 6th, 7th, 9th, and 11th days.	"	8	Ducts to lateral mass left free; 71 per cent of liver obstructed.	Before operation. After operation.	Bile pigment present for 5 days. Bile pigment present on 2nd, 3rd, and 12th days.	Negative. "
18	Normal dog. Fasting on 6th day only.	Bile pigment present on 6th and 7th days.	"	10	Ducts to left lateral lobe left free; 72.4 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 4th, 5th, 10th, and 12th days.	" "



12	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 4th, 9th, and 11th days.	Negative. " un-til 11th day, then faintly positive.
11	Duct to right lateral lobe left free; 82 per cent of liver obstructed.	Before operation. After operation.	Negative. Bile pigment present on 6th day.	Negative. "

concentrated failed regularly in our hands to give positive results with the plasmas of jaundiced dogs excreting cholates through the kidneys. This may have been due to the lack in the dog of a renal threshold for bile salts such as in man leads to their accumulation in the circulation.

### *The Renal Threshold for Bilirubin.*

In man, as is well known, the normal blood plasma has a yellow color owing to the presence of bilirubin; and a considerable increase in the pigment may occur without the passage of any into the urine, much less the appearance of a tissue icterus.<sup>15</sup> According to van der Bergh and Snapper<sup>11,16</sup> the plasma of healthy dogs is colorless and this has been our own finding except in animals with "physiological jaundice" induced by fasting. The plasma of normal monkeys is also in our experience practically colorless and fails to give the diazo reaction. We have further noted that in both dogs and monkeys bilirubinemia never occurs without bilirubinuria, while in dogs, at least, bile pigment is frequently to be found in the urine when it cannot be demonstrated in the circulation (Table I).

The state of the urine, then, furnishes a more delicate criterion of icterus in the dog than does that of the blood. The conditions are very different in man. In man, owing to the high renal threshold for bilirubin a considerable accumulation of pigment takes place in the blood before any escapes into the urine, and consequently one must reckon from a base-line of normal pigment retention in work upon defective bile elimination. This complicating factor does not exist in the dog and monkey. Such a renal threshold as is present in these animals—and *a priori* one would be expected on grounds of biological relationship—has so slight an elevation as to produce no evidence in the blood of its presence.

### *Method.*

The animals were kept in metabolism cages and the 24 hour urines were examined for a number of days prior to operation, as regards their general character and the presence of bile salts and pigments. The monkeys were males, but most of the dogs chosen were females and with one exception (No. 10) none was taken which

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<sup>16</sup> van der Bergh, A. A. H., and Snapper, J., *Berl. klin. Woch.*, 1914, li, 1109.

showed spontaneous icterus, so called. Throughout the work the routine tests were carried out upon cage urines, but positive findings were controlled by catheterization. At more or less frequent intervals, as occasion warranted, the blood was examined for bilirubin. The monkeys were fed on bananas, the dogs on bread and meat.

The biliary obstruction was induced by ligating and, where possible, cutting various branches of the hepatic duct under ether anesthesia. Asepsis was maintained, and the wound in the abdominal wall closed in three layers. When the free portion of the duct to be obstructed was too short to be doubly tied and cut, several stout ligatures were laid upon it, a procedure which in the dog generally served to close the duct throughout the term of experiment. Indeed, we have been but little troubled with the restoration of the duct channels by the cutting through of ligatures, possibly because of the large caliber of the silk used. In monkeys, on the other hand, even coarse ligatures were found to work through the duct walls within 10 days to 2 weeks, and either the continuity of the channel was reestablished, or a leak from it led to death. Needless to say, at every autopsy a careful study was made of the final results of operation and cultures were taken from the liver and the stasis bile. The animals were killed with chloroform. Instances in which infection existed were ruled from consideration.

#### *Arrangement of the Dog and Monkey Livers.*

The main liver mass of the dog consists of the left lateral and central and right central lobes with the ill defined quadrate lobe as an essential part of the last which also bears the gall bladder on its under surface.<sup>17</sup> There is another mass, separate both by contour and tissue cleavage, the lateral mass as we shall term it, which lies just above and to the right of the pylorus and is made up of the right lateral and caudate lobes. The main liver contains on the average about six-tenths of the parenchyma, the lateral mass about three-tenths. The remaining one-tenth consists of the papillary, or Spigelian, lobe placed below the main liver and separated from the lateral mass by the gastrohepatic omentum, but connected with both by tissue bridges. The course of the bile ducts falls in roughly with the anatomical arrangement of the tissue which they drain. A large branch coming from the left lateral and central lobes joins another from the right central and caudate lobes,—into which, by the way, the cystic duct opens,—and thus there is formed a large main channel into which the small papillary branch empties, and, lower down, within a few centimeters of the intestine, the considerable duct from the lobe mass. Frequent marked variations from this typical arrangement are encountered. Thus, for example, the branch from the lobe mass may course toward the main liver to join that from the right central and caudate lobes. Or the branch which appears to spring from the whole left central and

<sup>17</sup> Bradley, O. C., *A guide to the dissection of the dog*, London, New York, Bombay, and Calcutta, 1912.

TABLE II.  
*Weight of the Lobes of the Normal Dog Liver, Expressed in Percentages of the Total Organ.*

Dog No.	Body weight. kg.	Sex.	Liver weight. gm.	Papillary lobe. percent	Lateral mass.		Entire lateral mass. percent	Main liver.			Entire main liver. percent
					Right lateral. percent	Caudate. percent		Left lateral. percent	Left central. percent	Right central. percent	
1	10	M.	315.2	5.8			29.0				65.2
2			181.5	7.3			29.3	25.4	11.5	26.5	63.4
3	7 <sup>3</sup> <sub>4</sub>	M.	226.0	5.1	19.5	9.8	23.5	32.9	16.4	22.1	71.4
4	11	F.	330.5	6.1	14.8	8.7	31.6	24.5	13.6	24.2	62.3
5	15	M.	545.0	4.6	16.8	14.8	36.0	27.1	12.1	20.2	59.4
6	11		305.5	5.4	22.8	13.2	21.0	30.1	15.8	27.7	73.6
7	7	F.	212.5	4.7	12.4	8.6	34.0			19.1	61.3
8	11 <sup>3</sup> <sub>4</sub>	M.	505.0				40.0				60.0
9	8	"	279.0				27.0				73.0
10	9 <sup>1</sup> <sub>4</sub>	F.	288.0				22.7				77.3
11	11	M.	337.0	6.2			23.4			28.4	70.4
12	11 <sup>1</sup> <sub>4</sub>	"	423.0				27.1				72.9
13	8	"	253.5				21.0	37.0			79.0
14	9	F.	333.5				25.8	27.2			74.2
15	7	M.	242.0				31.0				69.0
16	7	F.	335.0				20.0			21.8	80.0
17	8	M.	359.0	5.0			33.2				61.8
18	10 <sup>1</sup> <sub>4</sub>	F.	359.5	4.6			32.0				63.4
19	9	"	351.0	6.0			27.1	25.0			66.9
20	8 <sup>3</sup> <sub>4</sub>	"	335.0	4.9			25.3	27.7			69.8
21	7	"	231.5	4.1			21.6	28.5	18.6	27.2	74.3
22	12 <sup>3</sup> <sub>4</sub>	M.	472.0	4.9			32.6	25.8			62.5
23	6	F.	285.0	4.6			36.8	24.5			58.6
24	8	"	252.5	3.9	21.6	14.5	36.1	27.7	11.7	20.6	60.0
25	6 <sup>1</sup> <sub>4</sub>	"	235.7	4.4	19.5	9.5	29.0	29.7	11.0	25.9	66.6

26	$6\frac{1}{2}$	F.	193.0	5.2	18.1	12.5	30.6	26.4	11.9	25.9	64.2
27	$7\frac{3}{4}$	M.	261.5	4.6	22.8	14.7	37.5	26.8	11.1	20.0	57.9
28	$8\frac{1}{2}$	"	311.0	5.8	15.4	12.2	27.6	25.0	9.5	32.1	66.6
29	$4\frac{1}{2}$	F.	138.5	5.1	15.8	13.8	29.6	25.9	13.1	26.3	65.3
Average .....											
				5.1	18.1	12.0	29.0	27.6	13.0	24.5	67.3
				(21 cases).	(11 cases).	(11 cases).	(29 cases).	(18 cases).	(12 cases).	(15 cases).	(29 cases).
Variation.....											
				3.9-7.3	12.4-22.8	8.6-16.9	20.0-40.0	24.5-37.0	9.5-18.6	19.1-32.1	57.9-80.0

lateral lobes may in reality drain but a small portion of these, their bile emptying for the most part into the just mentioned duct from the right central and caudate lobes. Large vasa aberrantia are frequent. They may be patent and filled with bile for several centimeters above their junction with the common duct, but stop short of the liver tissue or cease thereabouts to be patulous. A mistaken dependence upon such vessels for bile elimination has in some of our experiments led to the development of total obstruction when a partial one had been projected.

The liver lobes are in general defined by a cleavage of the parenchyma nearly to the hilum of the organ; and when there is but one duct from a lobe or group of lobes its obstruction leads to stasis throughout the tributary region. The proportion of the total liver affected can be readily determined under such circumstances. Potentially, at least, the case is different when there are two ducts or more to a lobe and one is left open. Under such circumstances relief may perhaps come to the area in stasis through newly opened channels into the unobstructed neighboring tissue. For it is well known that the bile canaliculi anastomose freely within the lobules. That no relief comes of a magnitude meriting consideration will be shown further on. The main difficulty lies in determining the exact amount of tissue in stasis.

Table II gives the weights of the liver lobes of twenty-nine normal dogs, expressed in percentages of the organ. In all cases the liver was removed before the blood had clotted. It will be seen that there is a rather large individual variation in the tissue distribution.

The monkey liver is divided into five lobes, much as in the rabbit, and these are grouped into a main liver and a lateral mass, which are connected at the base by a broad tissue bridge, as are the individual lobes also. A single short duct comes from each of the masses mentioned, and these unite to form an hepatic duct into which the cystic duct enters lower down, as in human beings. None of the ducts from the individual lobes save that from the caudate is accessible to ligation. This circumstance like that of the early cutting through of the ligatures has much hampered our observations.

### *"Physiological Jaundice."*

Investigators upon icterus in the dog agree that it is frequently encountered in mild form in animals that appear normal. Naunyn<sup>18</sup> showed, as far back as 1869, that fasting for 24 hours regularly leads in most dogs to the appearance of bile pigment and salts in the urine. The icterus is not dependent on increased concentration of the urine, though it is made more evident thereby. A bilirubinuria from fasting was a frequent complicating factor in our early experiments, occurring

<sup>18</sup> Naunyn, B., *Arch. Anat., Physiol. u. wissenschaft. Med.*, 1869, 579.

regularly in the Sunday to Monday 24 hour specimen of unoperated animals as a result of the small ration of the day first mentioned. When a full diet was provided on Sundays intercurrent icterus became rare and often was traceable to disease. It was never found as the result of anesthesia or of the trauma of operation. In monkeys a fasting icterus was not noted, although it is said to develop occasionally in human beings.<sup>3</sup>

In view of these facts it seemed wise to keep several normal animals under observation with the operated ones as a control to intercurrent manifestations, and this was accordingly done.

### *Results of Total Obstruction in the Dog.*

In sixteen dogs total obstruction was produced, sometimes by ligating and severing the common duct, but more frequently by cutting its large tributaries after their individual ligation. The tissue icterus that followed was never as pronounced as in human beings under similar conditions but in all cases was easily recognizable, while, in all, bile pigment became abundant in the blood and urine.<sup>19</sup> The tissue icterus was first visible on the 5th to 10th day, as yellowed scleras. The results in four of the sixteen dogs which were followed with special care have been tabulated (Table III).

The first sign of biliary obstruction was the appearance, as a rule, of pigment in the urine during the second or third 24 hours after operation but sometimes earlier. The delay is due, as Affanassiew showed,<sup>20</sup> to bile accumulation in the gall bladder and distended ducts: when he filled these reservoirs with wax at the time of operation icterus developed very much more promptly. Bilirubinemia was usually noted within 24 hours after bilirubinuria, and cholestes were recognizable in the urine on the 3rd or 4th day. At autopsy of the dogs, after 9 to 46 days of obstruction, a general tissue jaundice was regularly found, the liver being especially affected.

<sup>19</sup> We have since autopsied an animal in which after 9 days of total obstruction no tissue icterus was discoverable. Urine and plasma were markedly tinted with bilirubin. There is little doubt that tissue pigmentation would have occurred in a day or so more.

<sup>20</sup> Affanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

TABLE III.  
*Results of Total and Partial Obstruction in Dogs.*

Dog No.	Sex.	Findings prior to operation.	Observed after operation for	Weight of animal.		Liver portion and per cent obstructed.	Postoperative findings.		Remarks.
				Initial.	Final.		Bilirubinemia.	Urine.	
			days	kg.	kg.				
1	F.	Negative for 9 days.	26	10 $\frac{3}{4}$	11	Total obstruction.	Positive on 3rd day and after.	Pigment on and after 2nd day; salts on 3rd day and thereafter.	Scleral jaundice appeared on 8th day.
2	"	Negative for 10 days.	15	9 $\frac{1}{2}$	9 $\frac{1}{2}$	"	Positive on 2nd day and after.	Pigment on and after 2nd day; salts on 5th day and thereafter.	Scleral jaundice appeared on 5th day.
3	"	Negative for 7 days.	20	3 $\frac{3}{4}$	2 $\frac{1}{2}$	"	Positive on 4th day and after.	Pigment and salts positive on 3rd day and after.	Scleral jaundice appeared on 8th day.
4	"	Negative for 4 days.	13	8 $\frac{3}{4}$	8 $\frac{1}{2}$	"	Positive on 3rd day and after.	Pigment and salts on 3rd day and after.	Scleral jaundice appeared on 9th day.
5	"	Negative for 3 days.	26	10 $\frac{1}{4}$	10 $\frac{3}{4}$	All except lateral mass; 71 per cent.	None.	Negative.	
6	"	Negative for 4 days.	10	9 $\frac{1}{2}$	9 $\frac{1}{2}$	"	"	" save for salts on 3rd day.	
7	"	Negative for 7 days.	37	7 $\frac{1}{2}$	6 $\frac{1}{4}$	"	"	Negative.	
8	"	Slight bilirubinuria of unknown cause during 5 days.	36	7 $\frac{1}{4}$	8 $\frac{1}{4}$	"	"	Pigment and salts faintly positive on 2nd and 3rd days; salts positive on 16th, 20th, and 21st days.	Unoperated control animals showed pigment and salts likewise.



9	F.	Negative for 6 days.	23	5 $\frac{1}{2}$	5 $\frac{1}{2}$	All except left lateral lobe; 72.4 per cent.	None.	Pigment dubiously positive on two occasions.	Unoperated control animals showed pigment and salts likewise.
10	M.	Negative for 7 days.	34	17	16	" "	"	Pigment occasionally throughout.	
11	"	Negative for 6 days.	36	14 $\frac{3}{4}$	16 $\frac{1}{4}$	All except right lateral lobe; 82 per cent.	"	Salts from 7th to 14th days; thereafter, like pigment, negative.	Pigment once dubiously positive in urine.
12	F.	Negative for 7 days.	45	15 $\frac{3}{4}$	14 $\frac{1}{2}$	" "	"	Salts from 8th to 37th days; pigment on 4th, 9th, and 42nd days.	
13	"	Negative for 7 days.	15	9 $\frac{1}{2}$	9 $\frac{1}{2}$	All except papillary lobe; 95 per cent.	Positive on 11th day and after.	Pigment on 2nd, 3rd, and 4th days; salts and pigment on 11th day and thereafter.	
14	"	Negative for 7 days.	15	6 $\frac{3}{4}$		" "	Positive on 2nd day and after.	Pigment on 2nd day and after; salts on 1st day and after.	
15	F.	Negative for 8 days.	28			Control animal.	None.	Pigment on 18th and 20th days; pigment and salts on 22nd, 24th, and 25th days. Negative throughout.	
16	"	Negative for 4 days.	33			" "	"		
17	"	Negative for 5 days.	32			" "	"	Pigment on 11th, 12th, 13th, and 14th days; salts on 14th day.	
18	"	Negative for 5 days.	30			" "	"	Pigment and salts on 6th day and pigment on 19th day.	Pigment once dubiously positive in urine.

*Results of Partial Obstruction in the Dog.*

Partial obstruction was produced in ten dogs. Bile accumulation within these animals would presumably take place more slowly than on total obstruction, owing to the smaller amount of tissue in stasis and to the eliminative activities of the unobstructed portion. For these reasons no animal was considered to have given negative results as regards icterus until at least 10 days after operation and most were kept under observation for several weeks. As we shall point out further on, the unobstructed liver portion undergoes some hypertrophy within 10 days, while that in stasis atrophies, and thus the tendency to bile retention must soon be counteracted by lessened secretion in the region of stasis combined with increased facilities for elimination.

In four dogs approximately seven-tenths of the liver parenchyma was placed in stasis, in two others about three-fourths, in two, four-fifths, and in two about nineteen-twentieths. Table III summarizes the findings. Tissue icterus is not recorded because it was never observed, even when the eliminative burden had been thrown abruptly upon a mere twentieth of the liver.

In the four animals in which the duct from the lateral mass, draining on the average 29 per cent of the whole organ, was alone left open, bile pigment and salts were never demonstrable in blood or urine save on days when they were also present in control animals from the same intercurrent cause, namely, fasting. This was the case too when the free duct was that from the left lateral lobe containing 27.6 per cent of the tissue. A slightly greater degree of obstruction, produced by tying and cutting all the ducts except that to the right lateral lobe, or 18 per cent of the liver, resulted in the appearance of bile salts in the urine during the 2nd week after operation, occasionally accompanied in one animal by bile pigment as shown with the Gmelin test. The amount of pigment was always too slight to be quantitated, and bilirubinemia was not observed. Finally, when all the ducts were closed except that from the little papillary lobe, which holds from 3.8 to 7.3 per cent of the liver tissue, or on the average 5.1 per cent, pigment and cholates did indeed appear regularly in both blood and urine, and almost as rapidly as when total obstruction had been

produced. In the two instances studied a sufficient bile elimination took place, however, to prevent tissue icterus during the 15 days of observation, and in view of the papillary hypertrophy found at the end of this period there is but slight reason to suppose that a greater retention would have occurred later.

The four normal dogs that shared the general conditions of the operated animals and were followed in the same way yielded findings that were several times of great value, disclosing that bilirubinuria and choluria noted in the operated animals were "physiological," from food deprivation. In this connection it seemed of interest to determine whether animals with seven-tenths of the liver in bile stasis would show fasting icterus more readily than normal controls. Accordingly during a period of 6 days several animals of each sort were fed only a thin bouillon. This they took in quantity with result that the urinary output remained large. The experiment will be set forth in detail in a later paper on the physiology of jaundice. Here we shall merely state that while icterus appeared in most of the animals, it developed no sooner and was no more marked in the operated individuals than in the controls.

### *Liver Adaptation.*

In dogs with total biliary obstruction jaundice of the liver parenchyma is outspoken after a few days. By contrast, when obstruction is partial, even when it affects nineteen-twentieths of the organ, an hepatic icterus is not observed. From this it is evident that vicarious bile elimination becomes effective very close to the source, so to speak, a fact which is not surprising when one considers that the hepatic cells lie in the midst of copious blood and lymph streams, which, if kept free of bile, should keep them free also.

Long continued obstruction to the ducts from a part of the canine liver results in noteworthy changes in the whole organ, just as in the rabbit<sup>21</sup> and in man.<sup>22</sup> The portion in stasis gradually becomes sclerotic by an interlobular proliferation of the connective tissue, and the parenchymal cells undergo a gradual simple atrophy, and may

<sup>21</sup> Nasse, *Verhandl. deutsch. Ges. Chir.*, 1894, xxiii, pt. 2, 525.

<sup>22</sup> Carnot, P., and Harvier, P., *Arch. méd. exp. et anat. path.*, 1907, xix, 76.

TABLE IV.  
*Results of Total and Partial Obstruction in Monkeys.*

Monkey No.	Previous findings.	Observed after operation for days	Weight of animal.		Liver portion and per cent obstructed.	Postoperative findings.		Remarks.
			Initial.	Final.		Bilirubinemia.	Bilirubinuria.	
			gm.	gm.				
1	Negative for 13 days.	7	1,900	1,900	Total obstruction.	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 4th day and after.
2	Negative for 1 day.	8	2,375	2,300	"	Positive on 1st day and after.	Positive on 1st day and after.	Tissue icterus on 3rd day and after.
3	Negative for 1 day.	11	2,300	2,375	"	Positive on 2nd day and after.	Positive on 2nd day and after.	Tissue icterus on 3rd day and after.
4	Negative for 1 day.	12	3,100	2,900	"	Positive on 5th day and after.	Positive on 5th day and after.	At operation a pathological dilatation of the bile passages was noted. Tissue icterus on 8th day and after. Duct continuity restored toward end of experiment.
5	Negative for 5 days.	12	2,300	2,325	All except lateral mass and papillary lobe; 75 per cent.	None.	None.	
6	Negative for 21 days.	11	1,975	2,025	All except lateral mass; 80 per cent.	"	"	Questionable restoration of duct's continuity toward end of experiment.
7	Negative for 5 days.	11	2,650	3,225	"	"	"	Obstruction maintained.

ultimately disappear. Meanwhile, the tissue with duct unobstructed gradually hypertrophies. The underlying causes for these alterations have been analyzed in a previous paper from this laboratory.<sup>1</sup> In the dog they take place far more slowly than in the rabbit. A parenchymal shift is usually not discernible in less than 10 days, but after a month may be very marked. At this time the mass in stasis may be much shrunken, with finely hobnailed surface. Needless to say, instances free of infection are here alone referred to. The region with obstructed bile channels is now sharply demarcated from the adjacent hypertrophic tissue, and has been found to correspond closely in extent with the ramifications of these channels, thus proving that during stasis no important connections open between the blocked ducts and the neighboring free ones.

The shifting of tissue caused by local obstruction renders it impossible to determine exactly from the weights of the liver portions at autopsy how much of the parenchyma was originally placed in stasis. But the anatomical relations and a knowledge of the normal proportions of the liver lobes, such as Table II affords, enable one to reach an approximate conclusion on this point.

#### *Results of Biliary Obstruction in the Monkey.*

Seven monkeys were used (Table IV). They yielded results essentially similar to those in the dog. As in this animal, total obstruction was well tolerated, but bilirubinuria appeared more rapidly, developing in two out of four instances within the first 24 hours after operation. What would seem to be an interesting illustration of delay in its appearance owing to accumulation of bile in the ducts and gall bladder is afforded by Monkey 4. At operation a note was made that the bile passages of this animal, though undistended, were about three times the usual diameter. Despite the production of total obstruction, pigment failed to appear in urine or blood until the 5th day and tissue icterus was not seen until the 8th day. At autopsy all of the bile passages were enormously distended.

The plasma of monkeys subsisting on a banana diet is colorless and the urine nearly so. Bilirubinemia appears at approximately the

same time as bilirubinuria, on the 1st or 2nd day of total obstruction, and tissue icterus follows on the 3rd or 4th day. Cholates were never demonstrable in the urine by Hay's method. Further work would seem desirable on this point.

In three monkeys 75 to 80 per cent of the liver was placed in stasis. Owing to the rapidity with which ligatures cut through the ducts it was deemed best to terminate the experiments 11 and 12 days after operation. Even then in one instance there had been partial restoration of the duct continuity with some escape of bile into the intestine, though the well defined anatomical changes in the liver indicated that this was recent. At no time did the blood or urine of any of the animals contain bilirubin. That this would have continued to be the case is shown by the autopsy findings. For the livers, even in so brief a time, had undergone marked alterations in the direction of a functional readjustment. The unobstructed tissue was notably hypertrophied, and that in stasis shrunken and sclerotic. The changes had taken place almost as rapidly as in the rabbit, far more so than in the dog.

#### DISCUSSION.

The ability of a small portion of the liver to function for the whole as regards bile elimination when there is local obstruction depends without doubt upon several factors. That the parenchymal cells can rapidly excrete bile pigment and salts coming to them in large amount on the blood stream is proven by the promptness with which these substances pass into the bile when thrown into the circulation for experimental purposes.<sup>23</sup> Bile constituents formed by the tissue in stasis, and carried away from it by the blood and lymph, will of course be treated similarly. But the conditions of stasis themselves tend to lessen the amount of bile formed. For the obstructed ducts, dilating under pressure, interfere with the local portal flow<sup>24</sup> and divert a portion of the blood and with it the functional activities, to the more normal hepatic regions. There follows in due course a pa-

<sup>23</sup> Wertheimer, E., *Arch. physiol. norm. et path.*, 1892, iv, series 5, 577. Stadelmann, E., *Deutsch. med. W'och.*, 1896, xxii, 785.

<sup>24</sup> Betz, W., *Sitzungsber. k. Akad. Wissensch. Math.-naturw. Cl., Wien.*, 1862, xlv, 238.

renchymal shifting from the region in stasis,<sup>1</sup> which, as time passes, tends to become complete. It is possible, furthermore, that stasis directly influences the liver cells to form less bile, but this cannot be profitably discussed.

Attempts should be made to determine in human beings the margin of safety in bile elimination. Quantitative studies on the rate of disappearance of jaundice after the surgical relief of total biliary obstruction should provide interesting figures on the ability of the liver to excrete pigment in excess. Injections post mortem into the bile ducts to determine the degree of obstruction in congenitally cystic livers and livers with widespread carcinomatosis ought, in connection with the clinical findings, to yield data of value. Perhaps the most direct evidence, though, is to be had from cases of local obstruction by intrahepatic calculi.

Beer,<sup>25</sup> who described many instances of stones within the liver, remarks on the fact that even when numerous and widely distributed they often cause no symptoms during life. Lewisohn<sup>26</sup> tells of finding at operation a liver studded with fibrous nodules in each of which was one or more gall stones. Yet the patient had for a long time been in good health and unjaundiced. Carnot and Harvier<sup>22</sup> report the complete atrophy of a liver lobe as result of an intrahepatic calculus occurring in a patient who was never jaundiced. Such instances, as well as those more frequent ones of disseminated hepatic carcinomatosis without jaundice, which come under clinical observation, give good reason for the belief that the human liver possesses a margin of safety in bile elimination not inferior to that of the dog and monkey.

There is a point of immediate practical import in the demonstration that the biliary obstruction required to produce jaundice is one affecting the greater portion of the liver. Jaundice is not infrequently seen in association with abscesses and other less discrete inflammatory changes occupying but a small portion of the hepatic tissue. The inference from such observations has been that the jaundice results from local bile resorption. But in view of our

<sup>25</sup> Beer, E., *Med. News*, 1904, lxxxv, 202.

<sup>26</sup> Lewisohn, R., *Ann. Surg.*, 1916, lxiii, 535.

findings this would seem highly unlikely. Rather should one think in such instances of a general injury either to the liver parenchyma or ducts, or else to the blood corpuscles.

#### SUMMARY.

The bile ducts from three-quarters of the liver substance in dogs and monkeys can be obstructed without any clinical evidence developing of pigment or cholate accumulation in the organism. And in the dog nineteen-twentieths of the liver substance can be placed in stasis without the occurrence of tissue icterus such as regularly follows total obstruction in this animal. There is no reason to suppose that this will not be found true in the monkey as well. Always a local obstruction results sooner or later in atrophy of the affected tissue with compensatory hypertrophy elsewhere. Thus as time passes the derangement of function produced by the sudden stasis is progressively lessened.

The plasma of the dog and monkey, unlike that of man, is normally free from bilirubin, and this pigment so readily escapes from the blood into the urine that bilirubinuria is often to be found in the dog in the absence of bilirubinemia, while the latter is never met with alone in either animal. It follows that in both species the renal threshold for bilirubin is much lower than in man,—if indeed one can be said to exist at all.

The amount of biliary obstruction required to produce jaundice in human beings is probably as great as in the experimental animals with which we have dealt. The clinical jaundice encountered in association with local liver lesions should be viewed not as the result of local bile resorption, but as due to a general injury to the hepatic parenchyma or ducts, or to blood destruction.



# TUBERCULIN HYPERSENSITIVENESS IN NON-TUBERCULOUS GUINEA PIGS INDUCED BY INJECTIONS OF BACILLUS-FREE FILTRATES.

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(Received for publication, February 16, 1921.)

## INTRODUCTION.

The hypersensitiveness of the tissues of a tuberculous animal to tuberculin provides a means for the accurate determination of infection with *Bacillus tuberculosis* in man and domestic animals and it has led to much valuable experimental work to explain its mechanism. Koch's observation that tuberculous animals resist more or less successfully attempts at reinfection has been repeatedly confirmed, and since the introduction by Römer and Joseph<sup>1</sup> of the intracutaneous tuberculin test which is applicable to guinea pigs, evidence has been accumulating to show that there is a parallelism between intensity of the local response to tuberculin and resistance to reinfection. Baldwin<sup>2</sup> found that small injections of killed tubercle bacilli and various extracts containing the protein of the bacillus sensitize to tuberculoprotein so that anaphylactic shock may be elicited, but that such sensitized animals give no cutaneous response to tuberculin. Living virulent tubercle bacilli inclosed in Berkefeld filters and placed in the peritoneal cavity of guinea pigs did not lead to the development of a tuberculin reaction in the animals. In other experiments he found that repeated small injections of charcoal saturated with a filtrate of tubercle bacillus extracts, with a benzene

<sup>1</sup> Römer, P. H., and Joseph, K., *Berl. klin. Woch.*, 1909, v, 1300.

<sup>2</sup> Baldwin, E. R., *Tr. Nat. Assn. Study and Prevent. Tuberc.*, 1911, vii, 351.

extract containing tubercle bacillus wax, or with whole blood or sera from tuberculous animals resulted in no response to tuberculin. A number of these experiments have been repeated and extended by Krause<sup>3</sup> and by others. Krause summarizes the results in the statement that: "There is no cutaneous hypersensitiveness without a focus (tubercle)." It was found by the author<sup>4</sup> in the course of some experiments to determine the effect of non-pathogenic acid-fast bacilli on the blood leucocytes that injections into guinea pigs of large and small doses of living cultures of non-virulent tubercle bacilli (saprophytic) were not followed by a development of the tuberculin reaction.<sup>5</sup>

If the tuberculin reaction is dependent upon a hypersensitiveness to a foreign protein, then the failure of living tubercle bacilli (saprophytic) which have lost their virulence to bring about a tuberculin response means presumably that the protein of these bacilli has undergone an essential change. That killed cultures of virulent tubercle bacilli and various derivatives of these may not contain the identical protein present in living virulent ones and do not therefore sensitize to the virulent bacilli or their products seems not unlikely in view of the vigorous means required to kill organisms of the acid-fast group. No more definite statement in regard to the very general characters of protein sensitization is possible than that subsequent to the sensitizing dose some soluble substance passes out from the blood or focal area of tissue receiving the injection to all the cells and tissues of the organism with the result that the cells and tissues acquire after a certain time a new property which renders them susceptible to toxic injury when brought into contact with the same protein. In tuberculosis whether the substance passing out from the focus of infection, the tubercle, to give the tuberculin reaction is the protein of the

<sup>3</sup> Krause, A. K., *J. Med. Research*, 1916-17, xxxv, 21; *Am. Rev. Tuberc.*, 1919, iii, 153.

<sup>4</sup> McJunkin, F. A., *J. Med. Research*, 1921, xlii, 201.

<sup>5</sup> One of the so called saprophytic cultures was received from the Hygienic Laboratory at the University of Michigan through the courtesy of F. G. Novy, and the other from the University of Cincinnati through the courtesy of W. B. Wherry. Both grow rapidly on simple media and neither produces tuberculosis in guinea pigs.

virulent tubercle bacillus, a split-product of this protein, or some substance resulting from the reaction of the tissue cells in the tubercle is uncertain. However this may be, it seems likely that when virulent tubercle bacilli are injected into the peritoneal cavity of tuberculous guinea pigs a certain number of bacilli are broken up into granules and dissolved in much the same way as some of them are in tubercles, and that any further action on the protein of the virulent bacilli which may follow their disintegration and solution would be of the same general nature. Kraus and Hofer<sup>6</sup> state that such solution of tubercle bacilli follows their injection into the peritoneal cavity of tuberculous guinea pigs; probably the so called Much granules found in "cold" abscesses are evidence of the breaking up of tubercle bacilli.

#### *Technique of the Intracutaneous Tuberculin Test.*

In a small syringe provided with a 27 gauge  $\frac{3}{4}$  inch needle 0.02 cc. of tuberculin<sup>7</sup> is diluted up to 0.1 cc. with saline solution. The needle is inserted, preferably with the eye outward, into the shaved skin of the lightest colored portion of the abdomen and sufficient diluted tuberculin injected to raise a 1 cm. bleb. This amount, which is less than half that used by Römer and Joseph and by others tends to give a reaction more definitely localized than larger amounts. When introduced properly the point of the needle is distinctly seen and the liquid is forced out against considerable resistance, while if the needle is inserted into the subcutaneous tissue the liquid is easily forced out and the bleb which is formed does not remain long. The technique requires care in animals weighing less than 400 gm. In testing animals it is always advisable to inject the tuberculin into one or more normal guinea pigs of about the same age as controls. In the negative reactions the puncture is usually visible at the end of 24 hours but there is no redness or induration. In the lightest reactions ("lightly positive") recorded as positive there is a 1 cm. area of redness with slight swelling at the end of 24 hours. In the reactions recorded as "moderately strong" there is a 1 or 2 cm. area of local-

<sup>6</sup> Kraus, R., and Hofer, G., *Deutsch. med. Woch.*, 1912, xxxviii, 1227.

<sup>7</sup> The tuberculin (O. T.) was obtained from H. K. Mulford Company.

ized redness and distinct induration at the end of the 24 hours, while in the "strongly positive" reactions necrosis and sloughing take place. In the reactions characterized by necrosis a central opacity appears in 24 hours, and by the end of 48 hours hemorrhage into a necrotic center frequently occurs and an open ulcer follows. Lightly positive reactions have not been relied upon to any extent, but they appear to be trustworthy if careful controls in sufficient numbers are employed. Moderately strong responses also should be carefully controlled by simultaneous tests on normal guinea pigs. A guinea pig that has received an injection of virulent bacilli becomes positive in about 2 weeks.

#### EXPERIMENTAL.

The experiments consist essentially of the treatment of normal guinea pigs with a bacillus-free filtrate obtained from the peritoneal cavity and organs of tuberculous guinea pigs 6 to 24 hours after intraperitoneal injections of large amounts of heavy suspensions of living virulent tubercle bacilli.

In the first experiment two guinea pigs were injected with a bacillus-free filtrate, No. 23. To prepare this filtrate a 320 gm. tuberculous guinea pig which had been inoculated intraperitoneally 37 days before with Culture H37 and had shown a strongly positive reaction to tuberculin was injected intraperitoneally at 2 p.m. on Dec. 13, 1920, with the growth from a 6 week flask culture of Culture H37 in 20 cc. of glycerol bouillon. After the removal of all except about 5 cc. of the bouillon the growth was ground in a mortar and suspended in 16 cc. of 2 per cent gelatin. 5 p.m. Animal alive. Dec. 14, 9 a.m. Found dead. The peritoneal cavity was opened and 20 cc. of clear and somewhat viscid exudate were removed and the undiluted liquid was passed through a 2½ inch Berkefeld filter (N). The omentum was gathered in a caseous mass but the other organs showed no extensive lesions. A film of the unfiltered peritoneal exudate showed a few neutrophils and a few mononuclear cells. An occasional leucocyte of both varieties contained a few tubercle bacilli. There were present a few clumps of extracellular acid-fast granules and an occasional intracellular one, and although they appeared to be partially dissolved bacilli it is not possible to identify them positively as such. About 10 cc. of clear filtrate were obtained. The culture, No. H37, is a human type which grows well on Dorset's egg medium and on glycerol bouillon, develops a positive tuberculin reaction in guinea pigs in the usual time, and causes their death in 6 weeks to a number of months according to the size of the dose and the age of the animal.<sup>8</sup>

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<sup>8</sup> The culture was obtained from the laboratory of A. I. Kendall of Northwestern University Medical School where it is known as Culture H37.

*Guinea Pig 39.*—Weight 300 gm. Received a subcutaneous injection in the right groin of 1 cc. of No. 23 filtrate on Dec. 14, 1920, immediately after the filtration had been completed, 1 cc. on Dec. 17, and 2 cc. on Dec. 21. At this time a tuberculin test was made and the following day there was redness at the site of injection, but owing to a slight abrasion of the skin due to careless shaving this was not recorded as positive. Dec. 22. The test was repeated and gave a positive reaction the following day. Dec. 24. Test was recorded as moderately strong. Dec. 27. Tuberculin was again administered. Dec. 28 and 29. Test recorded as strongly positive. Dec. 30. A 1 cm. ulcer was present at the site of the last tuberculin injection. Dec. 31. The filtrate injections were discontinued. Jan. 6, 10, 12, and 19, 1921. The tuberculin test was moderately strong or lightly positive. Jan. 31. The test was negative. On Feb. 1, 49 days after the first filtrate injection, the animal, which then weighed 520 gm., was chloroformed.

*Autopsy.*—Nothing suggesting a tuberculous process was found. One or two translucent lymph nodes measuring 2 or 3 mm. were found in the superficial part of the groin fat. These as well as some of the deeper fibrous tissue were smeared on slides and stained by the Ziehl-Neelsen method; no acid-fast bacilli were found. No tubercles were visible to the naked eye in any of the organs and histologic examination of the omentum, spleen, liver, and lungs was negative.

*Guinea Pig 41.*—Weight 440 gm. Dec. 24, 27, and 30, 1920, and Jan. 1, 1921. Given 1 cc. of Filtrate 23. Jan. 1. A tuberculin test was negative. Jan. 3. Tuberculin was again tried and on Jan. 4 and 5 there were slight reddening and slight induration. Jan. 6. A similar response was obtained. These reactions were recorded as lightly positive. Jan. 8, 10, 19, and 26, 1921. The tests were negative. On Dec. 24, when the first injection was given, the filtrate, which had stood in the ice box for 9 days, was somewhat clouded and showed some bacterial contamination. Feb. 1. Animal weighs 660 gm. It is to be kept for a number of months.

In the second experiment four guinea pigs were injected with filtrates; two received a filtrate prepared in much the same way as that used for the injection of Guinea Pig 39 while the other two received a filtrate which was obtained by crushing the tissues of the abdominal wall and certain of the organs. These filtrates were obtained from Guinea Pig 40 which weighed 670 gm. and had developed a strongly positive tuberculin reaction 7 days after a large groin inoculation with Culture H37. The suspension of tubercle bacilli for injection into the peritoneal cavity of this tuberculous guinea pig was prepared in the same way as that used in the first experiment to obtain Filtrate 23, except that 30 cc. of 2 per cent gelatin were used to suspend the bacilli and all except about 1 cc. of the bouillon was removed. After 24 hours the animal was alive but appeared very toxic. It was killed with chloroform and the 15 cc. of exudate obtained from the peritoneal cavity were diluted with 15 cc. of saline solution and passed through a Berkefeld filter. The heart, lungs, kidneys, a portion of the liver, and muscle

from the abdominal wall were ground in a mortar, mixed with 50 cc. of saline solution, and the liquid was passed through another filter.

*Guinea Pig 46.*—Weight 460 gm. Injected in the groin with 6 cc. of filtrate obtained from the peritoneal exudate on Dec. 30, 1920, 4 hours after the animal had been chloroformed and the filtration started. On Jan. 1, 1921, injected with 2 cc., Jan. 3 with 1 cc., and Jan. 5 with 3 cc. Jan. 6. The tuberculin test was lightly positive. Jan. 10, 12, and 19. Test moderately strong. Jan. 26. The test was lightly positive.

*Guinea Pig 47.*—Weight 210 gm. Received the same filtrate on the same days as No. 46, but the quantity given was somewhat less and averaged 2 cc. per dose. The responses to tuberculin were the same.

*Guinea Pigs 48 and 49.*—Weight 260 and 220 gm. respectively. Received groin injections of 2 cc. of filtrate obtained from the crushed organs on Dec. 30, 1920, and 1 cc. on Jan. 1, 3, and 5, 1921. There was no response to tuberculin tests until Jan. 10 when both animals gave moderately strong reactions. Jan. 12. The same responses were present. Jan. 19 and 26. The tests were lightly positive.

*Autopsies.*—Feb. 1. Guinea Pigs 47 (weight 340 gm.) and 48 (weight 360 gm.) were chloroformed and films were made and examined after staining by the Ziehl-Neelsen method. In both these animals just beneath the groin fat a small bit of injected tissue was present. This slight local reaction was thought to be due to tuberculin which had been injected Jan. 31 and indicates that the tissues at the site of the filtrate injections are more sensitive than the skin a short distance removed. This reddened tissue was smeared out on slides and stained. No tubercle bacilli were found in these films or in those made from the small lymph nodes. Histologic examination of the omentum, spleen, lungs, and liver showed nothing which suggested tubercles. Guinea Pigs 46 and 49 are to be kept for a number of months.

In the third experiment (Table I) twenty-one guinea pigs were injected in the left axilla with filtrate as follows: Jan. 17, 1921, 2 cc. of Filtrate 45 (Table II); Jan. 19, 5 cc. of Filtrate 51; Jan. 21, 5 cc. of Filtrate 11; Jan. 24, 4 cc. of Filtrate 43. Animals 62, 63, 74, and 75 were placed in the series as controls and received no filtrate injections. In order to secure a more concentrated filtrate such as the one used for Guinea Pig 39 in the first experiment exceptions were made in six guinea pigs, Nos. 54, 55, 56, 67, 68, and 69, which in addition to the filtrate injections noted above were given on Jan. 27 injections of 5 cc. of Filtrate 80; on Jan. 29, 5 cc. of Filtrate 12; and on Feb. 2, 3 cc. of Filtrate 81. There was no demonstrable general or local reaction in any of the animals until the day following the fourth injection, Jan. 25, when a very slight amount of induration appeared in the axillary tissue. Feb. 3. Nos. 54, 55, 56, 67, 68, and 69 were the only ones that showed induration in the axilla and in these the reaction consisted of a slight and somewhat indefinite thickening of the axillary tissue. When the results of the tuberculin test were seen on Jan. 28 it was thought that the

dilution of the filtrate used in the first four injections was too great to secure the response obtained in Guinea Pig 39 which received about half of the total amount of filtrate obtained from one animal.

The filtrates for the third experiment were prepared from guinea pigs tabulated in Table II. The suspensions were made from a 3 to 6 week growth of

TABLE I.

Guinea pig No.	Weight.	Dates of tuberculin tests and responses.							
		Jan. 27.	Jan. 28 and 29.	Jan. 31.	Feb. 1 and 2.	Feb. 8.	Feb. 9.		Feb. 10 and 11.
	<i>gm.</i>								
54	310	Test.	±*	Test.	+	Test.	±	Test.	++
55	310	"	±	"	+	"	+	"	++
56	310	"	—	"	+	"	+	"	++
57	400	"	+	"	+				
58	320	"	±	"	+				
59	320	"	±	"	+				
60	340	"	±	"	+				
61	360	"	+	"	+				
62	360	"	—	"	—				
63	420	"	—	"	—				
64	400	"	+	"	+				
65	380	"	+	"	+				
66	400	"	±	"	+				
67	400	"	—	"	+	Test.	+	Test.	++
68	400	"	—	"	+	"	+	"	++
69	410	"	—	"	+	"	+	"	++
70	400	"	—	"	+				
71	340	"	±	"	+			Test.	—
72	330	"	±	"	++				
73	360	"	±	"	+				
74	340	"	—	"	—				
75	330	"	—	"	—			Test.	—
76	340	"	—	"	+			"	+
77	340	"	±	"	+				
78	360	"	—	"	+				

\* A lightly positive reaction is indicated by +, a moderately strong by ++, and a strongly positive by +++. Doubtful reactions are indicated by ±.

Culture H37 on about 20 cc. of glycerol broth contained in a 150 cc. Erlenmeyer flask. The growth was allowed to continue until a film spread over the entire surface of the bouillon; the contents of the flask were then placed in a mortar, much of the broth was removed, and the growth was ground for a few minutes. A variable amount of saline solution was used for making the final suspensions

TABLE II.

Guinea pig No.	Date.	Weight.	Treatment.	Tuber- culin test.	Filtrate obtained.
3	1920 Sept. 30	gm. 425	Intraperitoneal inoculation with Culture H37.	+++	Filtrate from incubated organs and bacillus suspension.
	Nov. 29	540	Heart's blood and organs removed aseptically.		
11	Oct. 5	420	Intraperitoneal inoculation with Culture H37.	+++	110 cc. of filtrate from peritoneal cavity and organs ground in mortar, which is about one-half the fluid used for dilution.
	1921 Jan. 20		20 cc. of suspension of Culture H37 injected intraperitoneally.		
	" 21		Dead.		
12	1920 Oct. 6	410	Intraperitoneal inoculation with Culture H37.	+++	30 cc. of filtrate from peritoneal cavity and organs, which is about one-half the fluid used for dilution.
	1921 Jan. 28		40 cc. of suspension of Culture H37 injected intraperitoneally.		
	" 29		Dead.		
23	1920 Nov. 6	320	Intraperitoneal inoculation with Culture H37.	+++	About 10 cc. of filtrate from undiluted peritoneal exudate.
	Dec. 13		16 cc. of suspension of Culture H37 injected intraperitoneally.		
	" 14		Dead.		
29	" 1	580	Subcutaneous inoculation with Culture H37.	+++	Filtrate from incubated organs and bacillus suspension.
	" 20		Heart's blood and organs removed aseptically.		
40	" 22	670	Subcutaneous inoculation with Culture H37.	+++	About 15 cc. of filtrate from peritoneal cavity and the same amount from the crushed organs; in each case this was about one-third the fluid used for dilution.
	" 29		30 cc. of suspension of Culture H37 injected intraperitoneally.		
	" 30		Killed with chloroform 21 hrs. after injection.		



TABLE II—*Concluded.*

Guinea pig No.	Date.	Weight.	Treatment.	Tuber- culin test.	Filtrate obtained.
	1920	gm.			
43	Dec. 28	640	Subcutaneous inoculation with Culture H37.		95 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	1921				
	Jan. 23		20 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 24		Killed with ether 23 hrs. after injection.		
	1920				
45	Dec. 28	610	Subcutaneous inoculation with Culture H37.		54 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	1921				
	Jan. 16		30 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 17		Killed with ether 21 hrs. after injection.		
51	" 5	720	Subcutaneous inoculation with dilute tuberculous sputum.		105 cc. of filtrate from peritoneal cavity and the organs, which is one-half the saline solution used for dilution.
	" 18		30 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 19		Killed with ether 23 hrs. after injection.		
80	" 16	640	Subcutaneous inoculation with Culture H37.		30 cc. of filtrate from peritoneal cavity and the organs, which is less than one-half the saline solution used for dilution.
	" 26		10 cc. of suspension of Culture H37 injected intraperitoneally.	++	
	" 27		Killed with ether 23 hrs. after injection.		
81	" 18	600	Subcutaneous inoculation with Culture H37.		15 cc. of viscid cloudy fluid were mixed with 25 cc. of saline solution used to wash out the peritoneal cavity. 18 cc. of filtrate obtained.
	Feb. 1		15 cc. of suspension of Culture H37 injected intraperitoneally.	+	
	" 2		Etherized 22 hrs. after injection.		

which were injected into the peritoneal cavity by means of a large gauge needle. In preparing the filtrates for the first and second experiments a 2 per cent gelatin instead of saline solution was used for suspension. As shown in Table II guinea pigs with a strongly positive tuberculin reaction die between the 8th and 21st hours following injections of the suspensions, while those with a less intense reaction live for a greater length of time. The peritoneal exudate was always mucoid in character and sometimes strikingly so. The amount, which was variable, was greatest when a large amount of suspension was injected. With single filtration through a fine filter the filtrate obtained in 4 to 7 hours was not more and often considerably less than one-half the volume of the combined saline solution used for washing out the peritoneal cavity and extracting the ground pulp of the organs. Filtration was usually started about 10 a.m. and finished between 1 and 5 p.m. The injections of the animals with the filtrate were made at once. Cultures of the filtrates on agar slants remained sterile. That the tuberculin test was positive and then became negative or of a less intensity in Guinea Pigs 39, 41, 46, 47, 48, and 49 seems to be conclusive proof that tubercle bacilli did not pass the filters. Guinea Pigs 52, 16, and 26 (see text below) received filtrates from similar heavy suspensions of tubercle bacilli and remained unresponsive to tuberculin.

*Guinea Pig 52.*—Weight 290 gm. Received 15 cc. of Filtrate 44 between Jan. 6 and 11, 1921, and gave repeated negative or doubtful reactions to tuberculin. Filtrate 44 was obtained from a 680 gm. guinea pig on the 7th day after a groin inoculation with Culture H37 when the tuberculin test was lightly positive. The amount of filtrate obtained was about 75 cc., which was less than one-third the amount of saline solution used for dilution. Jan. 28. The test was still negative.

Filtrates obtained by filtering mixtures of the incubated organs of two tuberculous guinea pigs and heavy suspensions of Culture H37 were injected into Guinea Pigs 16 and 26 but a typical response to tuberculin was not obtained. Guinea Pig 16, which weighed 400 gm., received on Dec. 21 to 30, 1920, four injections of 2 cc. each of Filtrate 29. This filtrate had been obtained by removing aseptically and grinding in a mortar the spleen, kidney, and 6 cc. of heart's blood of a tuberculous guinea pig with a strongly positive tuberculin reaction, mixing the paste with 10 cc. of a heavy suspension of Culture H37, incubating over night at 37°C., diluting with 20 cc. of saline solution, and filtering. Guinea Pig 26, which weighed 600 gm., received on Dec. 1 to 27, 1920, ten injections of 2 cc. each of Filtrate 3. This filtrate had been obtained from a tuberculous guinea pig with a strongly positive test by the same method used for No. 29 except that a tuberculous focus in the omentum was ground up with the organs. A typical response to tuberculin was not obtained.

## DISCUSSION.

A positive tuberculin test develops in about the same time after the filtrate injections as it does after inoculations with tubercle bacilli. The presumption is that the substance in the filtrate which leads to the tuberculin hypersensitiveness is identical with that present during the first few days in a tuberculous focus produced by a subcutaneous inoculation with living virulent tubercle bacilli and that it is a product of the action of the tissues of the tuberculous animal on the tubercle bacilli contained in the suspension introduced into the peritoneal cavity. There is some rather inconclusive microscopic proof that a disintegration and solution of some of the injected bacilli take place. In view of the many instances of the bacteriolysis of other bacteria under more or less analogous conditions this seems especially probable.

It has been shown by a number of investigators that guinea pigs hypersensitive to tuberculin are resistant to reinoculation. Experiments are now under way to determine whether the same is true of guinea pigs made sensitive to tuberculin by the injection of the filtrates. In order to induce an effective resistance to inoculation it seems reasonable to suppose that it will be necessary to establish by the filtrate injections a strongly positive tuberculin reaction. From the experiments it is evident that the substance in the filtrates which produced a hypersensitiveness to tuberculin when injected into normal guinea pigs varied quantitatively in the different tuberculous animals from which the filtrates were obtained. It appears that guinea pigs with a strongly positive tuberculin reaction as a result of peritoneal tuberculosis yield the most active filtrate.

## SUMMARY AND CONCLUSIONS.

1. When a guinea pig with well developed peritoneal tuberculosis is injected intraperitoneally with about 20 cc. of a heavy suspension of a virulent tubercle bacillus (Culture H37) death occurs within 24 hours or the animal becomes extremely toxic. Such a peritoneal tuberculosis develops in about 1 month after 1 cc. of a very heavy suspension of Culture H37 has been introduced into the abdominal cavity. If the viscid fluid which is contained within the peritoneal

cavity is mixed with saline solution and passed through a Berkefeld filter a bacillus-free filtrate is obtained which induces in normal guinea pigs a certain degree of cutaneous hypersensitiveness to tuberculin.

2. The abdominal organs and the parietal peritoneum, to which masses of leucocytes and tubercle bacilli are adherent, when crushed and extracted with saline solution yield a filtrate which likewise induces a cutaneous hypersensitiveness.

3. The cutaneous hypersensitiveness does not appear before the 7th or 8th day after the filtrate injection and is therefore considered to be the result of an active sensitization of the animal.

## STUDIES ON BACTERIAL NUTRITION.

### I. GROWTH OF *BACILLUS INFLUENZÆ* IN HEMOGLOBIN-FREE MEDIA.

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(Received for publication, March 8, 1921.)

Pfeiffer in 1892 first obtained growth of *B. influenza* by the use of agar slants, the surface of which was smeared with a few drops of human blood. Pfeiffer showed that this bacillus grew only in media containing blood or hemoglobin. The influenza bacillus was thus brought into the group of the hemoglobinophilic bacilli and has since always been considered an obligate hemophilic organism despite the fact that the literature shows that several investigators have been able to cultivate it in media free of blood or hemoglobin.

The first to show this fact was Cantani (1), who in 1901 obtained growth of *B. influenza* on ascites agar in symbiosis with other bacteria, such as gonococcus, diphtheria bacillus, and several large cocci. *B. influenza* grew in giant colonies in the vicinity of the other colonies. Cantani also obtained growth on the surface of agar to which had been added emulsions of bacteria killed by heat at 60°C. for 3 hours. Cantani excludes symbiosis and supposes that some factor in the bacterial cell induces the growth of *B. influenza* and that this factor is more easily liberated from the dead than from the living cell.

Neisser (2), however, demonstrated the growth of influenza bacilli in symbiosis with xerosis bacilli on plate cultures made from the inflamed conjunctivæ of a child. Impure colonies were observed which could be transferred through many generations on plain agar without the disappearance of *B. influenza*. The latter, however, failed to grow on this medium if the xerosis bacillus was not present.

Neisser also tried to cultivate *B. influenza* on media prepared by adding killed emulsions of the xerosis bacillus or the diphtheria bacillus to plain agar. A slight growth occurred for three or four generations, but the continued cultivation of *B. influenza* on this medium was impossible. He therefore considered the growth of *B. influenza* in mixed cultures as a symbiotic phenomenon.

Grassberger (3) observed that when *B. influenza* was grown on blood or hemoglobin agar plates in association with other bacteria, especially staphylococci, the colonies of influenza bacilli adjacent to the colonies of cocci were of unusually large size. He assumed that the cocci exerted some effect upon the blood medium that was beneficial for the growth of *B. influenza*.

Luerssen (4) confirmed the observations of Cantani by growing *B. influenza* on agar containing emulsions of dead staphylococci, *B. coli*, or *B. prodigiosus*. Growth of *B. influenza* did not occur in ordinary mixed cultures with these other organisms. The bacterial emulsions were sterilized by heating at 60°C. for 3 hours; if they were boiled the growth which occurred was not so good. Luerssen presumes that the bacteria contain a factor that exerts a stimulating effect upon the growth of *B. influenza*, and that this is destroyed at high temperatures. He found that this growth factor is contained in the bacterial cell, since the carefully washed cells are still active. Growth of *B. influenza* did not occur if the enriching emulsion of dead organisms was smeared on the surface of the agar; it only occurred if the emulsion was incorporated in the medium. Luerssen also observed that *B. influenza* grew sparsely in sterile filtrates of broth cultures of staphylococci, *B. diphtheria*, and *B. violaceus*.

Ghon and von Preyss (5) attempted to grow *B. influenza* in media containing no hemoglobin, but the results were negative and they concluded that the reason other investigators had been successful was that in making the inoculations small amounts of blood had been carried over. They likewise held the opinion that when *B. influenza* grows on plain agar, as occasionally happens, this agar contains traces of hemoglobin.

Recently Putnam and Gay (6) tried to confirm the experiments of the earlier investigators. They were unable, however, to obtain any growth of *B. influenza* on plain agar to which either killed or living cultures of *B. xerosis*, *B. diphtheria*, *B. coli*, or staphylococci had been added.

In spite of the observations concerning the growth of *Bacillus influenza* in media free of blood or hemoglobin, the opinion that this organism is hemoglobinophilic has not been altered, and for its cultivation media containing blood or hemoglobin in some form is always employed.

#### EXPERIMENTAL.

In a study on the growth of mucoid bacilli and the transformation of non-mucoid bacilli into mucoid ones (7) the writer undertook the cultivation of different microbes, *Bacillus paratyphosus* B and pneumococci, in broth containing mucus produced by mucoid bacilli such as Friedländer's bacillus, the ozenabacillus, and other similar organisms. An attempt was also made to cultivate *Bacillus influenza* in this medium. The development of this work led to the present studies on bacterial nutrition.

*Experiment 1.*—A culture of a mucoid Gram-negative bacillus (Friedländer's) was grown on plain agar. After 24 hours in the incubator the growth from each plate was suspended in a few drops of plain broth. 0.5 cc. of this emulsion was added to 5 cc. of plain broth, then heated for 1 hour at 60°C., tested for sterility, and stored in the ice box. The reaction of the broth, pH 7.8, was not altered by the addition of the bacterial emulsion. With the medium prepared in this manner, the following tests were made.

A tube of this medium was inoculated with influenza bacilli from a blood broth culture, the blood cells of which had completely settled to the bottom of the

TABLE I.

*Growth of B. influenza in Plain Broth Added to Emulsions of Friedländer's Pneumonia Bacillus.*

No. of transfers from original culture in emulsion broth.	Age of culture when transferred.	Growth of culture on blood agar after standing in incubator.									
		No. of days.									
		1	2	3	4	5	6	7	8	9	10
Emulsion broth 1	hrs.										
	48	++	++				0				0
" " 2	days										
	4				+						+
" " 3	hrs.										
	24	++							+		
" " 4	hrs.										
	48	++			++			++			
" " 5	hrs.										
	48	++			++						0
" " 6	days										
	5	++	++								0
" " 7	hrs.										
	24	++			++						
" " 8	hrs.										
	48	++			++						
" " 9	Not transferred.	++									

tube. The following day 0.2 cc. of this subculture was transferred to another tube of emulsion broth. Simultaneously a loopful of the first culture was streaked on the surface of blood agar to demonstrate the growth of *B. influenza*. The last procedure was necessary since the emulsion broth in itself was too cloudy to show growth. From the first culture there were made eight successive transfers to emulsion broth, in all nine transfers from the original blood broth culture.

The result of these experiments demonstrates that the influenza bacillus grows well in the emulsion broth described. In this medium *B. influenza* was found living and capable of multiplying after 10 days at 37°C. (Table I).

Experiment 1 was repeated in emulsion broth prepared in the manner described from the mucoid growth of an organism classified as *Bacillus ozanæ*.

In this experiment the growth was washed off the surface of plain agar with 1 cc. of normal saline solution and the bacteria were killed by heating at 70°C. for 1 hour. 0.5 cc. of this sterile emulsion was then added to 5 cc. of plain broth. The emulsion broth was inoculated with 0.1 cc. of a blood broth culture of *B. influenza* after all blood cells had settled to the bottom of the tube. 24 hours later 0.1 cc. of this first emulsion broth culture was transferred to a second emulsion broth tube and simultaneously streaked on the surface of blood agar to determine growth. This series of cultures was carried on successfully for ten transfers in emulsion broth, and then was voluntarily discontinued. The ten consecutive cultures all showed typical colonies on the blood agar, and films of the last seven transfers showed typical bacilli and a few involution forms of *B. influenza*.

TABLE II.

*Determination of Smallest Amount of Emulsion Capable of Stimulating Growth of B. influenza.*

Tube No.	Amount of broth.	Amount of emulsion.	Growth.
	cc.	cc.	
1	5	0.5	+++
2	5	0.3	+++
3	5	0.1	+++
4	5	0.01	+
5	5	0	—

*Experiment 2.*—After it had been shown that an emulsion of heat-killed mucoid bacteria, when added to plain broth, is able to support growth of the influenza bacillus, it seemed desirable to learn how small an amount of the emulsion would suffice for this purpose. Accordingly, dilutions of the emulsion in broth were made and inoculated with comparable amounts of a culture of influenza bacillus. After 24 hours incubation subcultures were made on blood agar to confirm growth in the cultures containing various dilutions of the emulsion. These results are recorded in Table II.

0.01 cc. is evidently the lower limit of the growth-stimulating substance in this particular emulsion of heat-killed mucoid bacilli, since cultures containing this amount showed less growth than those in which larger quantities were used.



*Experiment 3.*—In order to determine whether the growth of *B. influenza* in the emulsion broth was more pronounced in the bottom of the tubes or at the surface of the broth, tubes were inoculated after all mucoid material had settled to the bottom. They were allowed to stand in the incubator for 24 hours and subcultures were made both from the thick residuum in the bottom of the tubes and from the superficial layers of the broth. The growth was compared with the following result.

	Growth.
(a) From bottom of tubes.....	+++
(b) From surface.....	+

The fact that this accessory substance appeared to be more concentrated in the immediate vicinity of the sedimented bacterial emulsion than in the upper portions of the culture fluid seems to indicate that the growth-stimulating factor is contained within the bacterial cell and slowly passes out into the surrounding fluid.

It therefore seemed reasonable to attempt to extract this substance from the bacterial emulsion. The addition of a clear bacterial extract to media, moreover, would have the advantage of making it possible to observe bacterial growth directly without the secondary transfer to blood agar. This was done in Experiment 4.

*Experiment 4.*—The growth of a mucoid organism was collected from agar plates and emulsified in plain broth, 1 cc. of broth being used to each plate. The emulsion was boiled for 5 minutes, and then centrifuged to separate the clear fluid extract from the bacterial bodies. This sterile extract was then tested for growth-stimulating action by the addition of decreasing amounts to plain broth. The medium prepared in this manner was inoculated with one drop from an emulsion broth culture (Table I, No. 6). The results of this experiment are recorded in Table III.

TABLE III.

*Growth-Inducing Action of an Extract of Mucoid Bacteria on B. influenza.*

Amount of extract.		Growth
cc.	per cent	
0.3	6.0	++
0.1	2.0	++
0.05	1.0	+
0.01	0.2	—
0.001	0.02	—
0	0	—

It is evident from Table III that it is possible by simple boiling of an emulsion of mucoid organisms to obtain an extract which when added to plain broth is capable of inducing growth of *Bacillus influenzae*. That the first extraction of the bacillary emulsion does not completely exhaust it of this growth factor is shown in the following experiment.

*Experiment 5.*—An emulsion of mucoid bacilli was made as previously described. A portion of this emulsion was heated at 60°C. for 1 hour and another portion was boiled for 5 minutes and then centrifuged and the clear supernatant extract pipetted off. The bacterial residuum was then washed in normal saline solution three successive times and the following experiment carried out.

	Growth.
5 cc. of plain broth + 0.5 cc. of unboiled emulsion heated to 60°C. for an hour.....	+
5 cc. of plain broth + 0.5 cc. of extract from boiled emulsion...	++
5 cc. of plain broth + 0.5 cc. of residuum from boiled emulsion.	++
5 cc. of plain broth (control).....	—

In this experiment the extract and the residuum from the boiled emulsion, when added to broth, gave even better growth than the emulsion heated at 60°C. for 1 hour.

That extraction of the growth-inducing substance is obtained simply by allowing the bacterial cells to remain in contact with broth for some time is shown in the following experiment.

*Experiment 6.*—To two tubes of plain broth there was added 0.5 cc. of a bacillary emulsion which had been heated to 60°C. for 1 hour. The tubes were then left in the ice box for 1 week. After this time, one tube was centrifuged and the clear supernatant fluid used as culture medium, while from the other, the supernatant fluid was pipetted off without being centrifuged. The two tubes were inoculated with 0.1 cc. of an emulsion broth culture of *B. influenzae*. Good growth occurred in both tubes.

This experiment indicates that the growth-inducing substance passes from the bacterial cells into the surrounding fluid and there exists apart from the cell.

In the foregoing experiments it has been shown that it is possible to obtain good growth of *Bacillus influenzae* in plain broth to which emulsions and extracts of mucoid bacteria have been added. It seemed reasonable, therefore, to seek the same growth factors in other microorganisms, and *Bacillus proteus* was selected as an organism which normally shows an abundant growth on ordinary media.

*Experiment 7.*—Agar plates were inoculated with *B. proteus*. After 24 hours growth, to each plate 1 cc. of normal saline solution was added, and the growth washed off and collected in a sterile centrifuge tube. The emulsion was boiled for 5 minutes. The sterile emulsion was then centrifuged and the supernatant fluid pipetted off. This extract was clear, yellowish in color and had a reaction of pH 7.6. 0.5 cc. quantities of this extract were used to enrich plain broth. After the addition of this amount the beef infusion broth remained clear, so that eventual growth could be indicated by the turbidity of the medium. Control cultures, however, were always made from the extract broth on blood agar or oleate hemoglobin agar, and in plain broth.

In the fluid medium thus prepared the following experiment was made. From a 24 hour culture of *B. influenza* in blood broth 0.1 cc. was transferred to *Proteus* Extract Broth 1; from this after 24 hours growth, to No. 2, and from this to No. 3. These three cultures all gave good growth and upon transfer to blood agar showed the typical colonies of *B. influenza*. Films also showed the typical small Gram-negative bacilli.

The experiment was voluntarily discontinued after the third transfer.

This experiment showed that *Bacillus influenza* would grow on a watery extract of *Bacillus proteus* for at least three generations. An experiment was next made with the whole bacterial emulsion of *proteus* in the same manner as described in Experiment 1. Here, however, growth of *Bacillus influenza* occurred only in the first two transfers after the blood broth culture. A considerable difference between the emulsions of the mucoid bacilli and the emulsion of *proteus*, therefore, seemed to exist. The explanation of this has not been found as yet, but it is reasonable to seek this in the morphological difference between these two microbes. The large capsule of the mucoid bacilli may be a better growth-inducing factor than the capsule-free *proteus*. The possibility that the capsule may contain some nutritional reserve for the bacillus has already been put forth by Toennissen (8), who finds that the capsule consists of a polysaccharide, galactan, and that other bacteria grow better on the surface of cultures of Friedländer's bacillus than on plain agar.

Since it had been shown that it was possible to extract a growth-inducing factor from *Bacillus proteus*, tests were made to determine the influence of this extract upon the growth of *Bacillus influenza* in various sugar solutions (1 per cent of sugar in peptone water with Andrade indicator (9)).

*Experiment 8.*—To each 5 cc. of sugar medium was added 0.2 cc. of *proteus* extract. The tubes were then inoculated with 0.1 cc. of culture (No. 2 in the foregoing experiment) and incubated. The following sugars were used: lactose, mannitol, maltose, dextrose, saccharose, raffinose, inulin, and salicin. After 12 hours incubation a heavy clouding of the medium was visible in all tubes and the dextrose culture had turned slightly red. After 24 hours the culture containing dextrose was distinctly red, while those containing the other sugars remained colorless. At this point, control cultures on blood agar from all tubes showed pure growth of *B. influenzae*. 4 days after inoculation the tubes showed the same reactions. On transfers to blood broth the cultures were all found to be living and pure.

*Experiment 9.*—It was considered of interest to determine whether or not the clear extract of *B. proteus* could be filtered through a Berkefeld filter without losing its potency. After the extract had been prepared as already described, it was passed through a Berkefeld filter N and the water-clear filtrate, after being proved sterile, was added to plain broth in the following amounts.

Broth.	Filtrate.	Growth.
cc.	cc.	
5	1.0	+++
5	0.5	+++
5	0.2	+
5	0.05	—
5	0.01	—
5	0	—

Growth was controlled by turbidity of the medium, by films, and by subcultures on blood agar and plain agar. These controls showed the growing organism to have the characters of *B. influenzae*.

This experiment shows that the bacillary extract in question can pass through a Berkefeld filter without losing its growth-inducing property.

#### SUMMARY.

From the data presented in the foregoing experiments it is evident that *Bacillus influenzae* will grow in a fluid medium consisting of plain broth to which have been added small amounts of emulsions or extracts of mucoid bacilli or of *Bacillus proteus*. The bacterial extracts may be made by simple boiling of the bacillary emulsions in broth or saline solution and centrifuging out the bacterial bodies; they may be filtered without losing their growth-inducing property.

Cultures of *Bacillus influenzae* in bacterial extract broth, if not too small doses of the extracts were employed, always showed heavier growth than the control cultures in blood broth, and growth occurred at a considerably earlier period than in blood broth. In many instances growth could be seen after 3 to 4 hours, and a bacterial whirl was always visible after 6 hours incubation.

When the nature of the culture used for seeding is not stated, this was 0.1 cc. of the supernatant fluid of a blood broth culture.

All cultures were made in fluid medium; solid medium is much more difficult to use in connection with the extracts.

In explanation of the remarkable growth of *Bacillus influenzae* in this blood-free medium, the idea is proposed that the growth-stimulating effect of the bacterial extracts is due possibly to substances of the same nature as the so called vitamins.

Further investigations on this principle of bacterial nutrition will appear in subsequent papers, together with a more thorough study of the sources and character of the growth-inducing substances.

#### CONCLUSIONS.

1. It is shown that *Bacillus influenzae* will grow profusely in hemoglobin-free media consisting only of plain broth and emulsions or extracts of mucoid bacilli and *Bacillus proteus*.

2. The emulsions and the extracts can be boiled and filtered through Berkefeld filters without losing their growth-inducing property.

3. The growth-stimulating effect of the bacterial extracts is possibly due to substances belonging to the class of the so called vitamins.

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## DISSOCIATION OF MICROBIC SPECIES.

### I. COEXISTENCE OF INDIVIDUALS OF DIFFERENT DEGREES OF VIRULENCE IN CULTURES OF THE BACILLUS OF RABBIT SEPTICEMIA.

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(Received for publication, February 24, 1921.)

#### INTRODUCTION.

It is a general rule to regard the individuals comprising a given strain of a microbic species as identical in nature. So, when a culture is spoken of as virulent, it is supposed to be virulent as a whole, and little or no attention is paid to its individual members. They are tacitly assumed to possess this character in common. As a culture attenuates by passage on artificial media, the formerly virulent germs are again assumed to lose their invasive property simultaneously and uniformly.

This point of view persists in spite of numerous facts that show the assumption of a community of characteristics to be without foundation. Chauveau and Phisalix (1) demonstrated atypical forms of *B. anthracis* in cultures from the lymph glands of animals that had died of this infection. Their *bacille en clou* possessed a morphology distinctly different from that of the typical organism. It was completely avirulent. Elser and Huntoon (2) found that colonies of different appearance existed side by side in freshly isolated cultures of *Micrococcus catarrhalis*, and that one type, increasing with each passage, tended finally completely to supplant the other. Bordet and Sleswijk (3) found an interesting variation to occur in the behavior of *B. pertussis*. When freshly isolated, this organism grew only upon media enriched with blood, but after several passages it became able to multiply upon ordinary media. The blood microbe had antigenic characters distinctly different from those of the agar microbe. Von Lingelsheim (4) discovered that variants having a peculiar type of colony formation arise in laboratory cultures of *B. typhosus*. His work has been elaborated and

extended to other members of the colon-typhoid group by Gildemeister (5). Baerthlein (6) has demonstrated the simultaneous presence, in single cultures of *B. pyocyaneus*, *Staphylococcus aureus*, and *B. coli*, of individuals giving rise to colonies of different appearance. The "H" and "O" forms of *B. proteus*, first described by Weil and Felix (7), are well known. Eisenberg (8) and Wagner (9) have studied thoroughly the presence of variants in cultures of *B. anthracis*.

The researches of Bordet on phagocytic reactions *in vivo*, following injections of *Vibrio metchnikovii* (10) and of streptococcus (11), have brought out with striking clarity differences in virulence existing among the individuals of one culture. It is remarkable that little stress has been laid on findings of such significance.

With this exception, differences of virulence have received little attention in the above mentioned studies. It has been our good fortune, in the course of studies on the rabbit septicemia bacillus, to observe wide variations in this characteristic, associated with definite difference in growth on fluid media and in colony formation. If all the organisms in these cultures had possessed indistinguishable growth characters, little progress could have been made. As it is, the association of a readily observable criterion with the virulence variation has greatly simplified the study of this problem.

#### *Origin of Materials.*

The strains of the rabbit septicemia bacillus used were isolated from spontaneous infections occurring among the normal animal stock of this laboratory. Eight strains were obtained in pure culture from the heart's blood at necropsy. The pathologic picture in six of these cases was that of bronchopneumonia, fibrinopurulent pleuritis, and pericarditis. In the remaining two no gross lesions of any kind were discernible, but the organisms were present in large numbers in the heart's blood.

#### *Method of Isolation.*

The heart's blood obtained at necropsy was planted in 10 per cent defibrinated rabbit blood broth. After incubation for 24 hours the cultures were plated out in 5 per cent rabbit serum agar. Isolated colonies were fished to rabbit serum broth. After incubation, growth, and microscopic study, this culture was streaked on 10 per cent rabbit serum agar slants, incubated for 10 hours, and then stored in the ice



chest for 7 days, when it was again transplanted. This routine was rigorously observed for all strains.

### *Characteristics of the Organism.*

The organism under investigation is without doubt one of the *pasteurella*. It is probably identical with the bacillus of rabbit septicemia first accurately described by Theobald Smith (12). It is a minute non-motile bacillus, occurring singly or in pairs. It is frequently coccoid in shape and in fresh preparation might be mistaken for pneumococcus. It is Gram-negative when stained by Stirling's method. Its bipolar nature is best demonstrated in films from the heart's blood, fixed by heat, and stained for 5 minutes in the cold with Loeffler's methylene blue. The microbes in the films from pleural fluid, tissues, and cultures tend to stain solidly. Pleomorphism is most marked in the condensation water of serum or plain agar slants.

Beef infusion broth and beef infusion agar, pH=7.4, fractionally sterilized, were used as base medium throughout the investigation. All the strains are strongly aerophilic. When first isolated from spontaneous infections they grow very feebly or not at all on ordinary media, but quite abundantly on serum agar or in serum broth. After several transplants on blood or serum agar, good growth occurs on ordinary media, provided that a sufficient quantity of material is carried over. The surface colonies on agar are circular in shape, with even borders. They are whitish and rather opaque at the center, more translucent toward the edges. They are strongly fluorescent, both in daylight and by artificial illumination. In serum and in plain broth the growth is uniformly turbid with little tendency to sedimentation. In tubes of serum broth, inoculated with 0.05 cc. of a 16 hour culture, the lag period is very short and the peak of growth reached by 8 hours incubation. From that time on a rapid decrease in the number of viable organisms occurs.

There is no visible growth on potato. Carbohydrates do not enhance growth. The strains isolated in this laboratory ferment dextrose, levulose, saccharose, and xylose with acid but no gas. The medium used was sugar-free broth to which 1 per cent of carbohydrate was added. The pH before inoculation was 7.5. After incubation at 37°C. for 66 hours, the levulose, dextrose, and saccharose tubes were found to have reached an acidity of pH=5.9 to 6.1. No acid formation was observed in maltose or lactose. In fact, definite alkalinity increase was observed with lactose—pH=8.2. Three strains received through the kindness of Dr. N. S. Ferry behaved as did those just described, with the exception that maltose was fermented.

## EXPERIMENTAL.

In the course of virulence studies with strains isolated about 6 weeks previously, the approximate number of organisms injected into the test rabbits was controlled by plating high dilutions of the test culture. Dilutions corresponding to  $10^{-9}$ ,  $10^{-8}$ , and  $10^{-7}$  cc. of the serum broth cultures under test were plated in 5 per cent rabbit serum agar. It was thought that plain agar might serve as well for this procedure, since all the strains under test had been growing for some time in artificial media. But upon seeding such dilutions

TABLE I.

*Growth of Dilutions of Serum Broth Culture R 15 in Serum and Plain Media.*

Dilution of original culture.	No. of colonies on plain agar plate.	No. of colonies on serum agar plate.	Growth in plain broth.	Growth in serum broth.
cc.				
$10^{-3}$	0	Countless.	—	—
$10^{-4}$	0	"	—	—
$10^{-5}$	0	"	+	+
$10^{-6}$	0	1,500	0	+
$10^{-7}$	0	194	0	+
$10^{-8}$	0	21	0	+
$10^{-9}$	0	2	0	+

+ indicates growth; 0, no growth; —, culture not made.

into plain agar plates, no growth occurred, despite the fact that animals injected with the same dilutions succumbed with typical infections. It was known that good growth could be obtained upon plain agar when a transplant was made of fairly large amounts from tube to tube. It was considered of importance to find out how many organisms it would be necessary to plant in order to obtain growth in medium without serum.

*Experiment 1. Growth of Dilutions of a 12 Hour Serum Broth Culture in Serum and Plain Agar and Serum and Plain Broth.*—A 12 hour 5 per cent rabbit serum broth culture of Strain R 15 was diluted with plain broth, pH=7.4, to  $10^{-9}$  cc. of the original culture. The dilutions were made as follows: Dilution A, 1 cc. of culture + 4 cc. of broth; Dilution B, 0.5 cc. of Dilution A + 4.5 cc. of broth; Dilution C, 0.5 cc. of Dilution B + 4.5 cc. of broth; and so on, to Dilution I, 0.5 cc. of which would be equivalent to  $10^{-9}$  cc. of the original. Different pipettes

were used for making each dilution. As soon as the dilutions were completed, and starting at dilution  $10^{-9}$  cc., 0.5 cc. was carried over into parallel large tubes of plain and of 10 per cent horse serum agar, which had been melted and cooled to  $45^{\circ}\text{C}$ . The tubes were carefully shaken and the contents poured at once into plates. Such plates were made from all dilutions, from  $10^{-9}$  down to  $10^{-3}$  cc. At the same time, inoculation of the same amount of material was made into parallel series of serum and of plain broth tubes, from dilutions  $10^{-9}$  to  $10^{-5}$  cc. The plates and tubes were incubated at  $37^{\circ}\text{C}$ . for 48 hours. The result is given in Table I.

Table I shows that no growth occurred in plain agar plates, even when these were seeded with  $10^{-3}$  cc. of the culture, an amount containing millions of organisms. Colonies appeared on all the serum agar plates, and in all the serum broth tubes. On the other hand, the plain broth series showed multiplication in  $10^{-5}$  cc., but not in higher dilutions. The most striking fact observed was the character of the growth in the plain broth subculture from dilution  $10^{-5}$  cc. Instead of growing diffusely with a uniform turbidity, as was the case with all the serum broth subcultures, the growth in the plain broth tube was granular in character. These granules were apparent even in early phases of the growth. They settled rapidly to the bottom of the tube, so that in 24 hours the supernatant fluid was perfectly clear.

Repetition of this experiment led to the same result. This phenomenon can be explained in one of two ways. First, it might be conceived that organisms growing diffusely in serum broth subcultures might flocculate when cultivated in plain broth. Or, second, two types of microbe might be supposed to exist in the same culture, one, preponderatingly present, and capable of growing only in serum broth, when seeded in small amount, the other, less numerous, sedimenting in fluid medium, and able to grow in plain broth, even when seeded in relatively small amount. To resolve this question the following experiment was undertaken.

*Experiment 2. Persistence of the Growth Character of Granular and Diffuse Growing Microbes.*—The plain broth subculture, dilution  $10^{-5}$  cc., of Experiment 1 grew in rapidly sedimenting, granular manner. This culture was seeded in amounts of 0.05 cc. to parallel tubes of plain and of 5 per cent rabbit serum broth. The procedure was repeated through six transfers. The granular growth persisted, in the serum as well as in the plain broth tubes. On the other hand,

it will be recalled that the serum broth subculture, dilution  $10^{-9}$  cc., of Experiment 1, exhibited a diffuse growth. This culture, seeded in similar amount into parallel serum and plain broth tubes, continued to grow diffusely in the plain as well as in the serum broth series.

It would seem, then, that the first of the two possibilities suggested, *i.e.* that of a change of growth character following transfer from serum to plain broth, is answered in the negative. On the other hand, the possibility still remained that the sudden transfer from serum to plain broth might cause a mutation from diffuse to granular growth character, which might persist even after return to serum broth. To rule out this contingency, it was necessary to determine other differences between the granular and diffuse growing microbes, which might lead to the demonstration of their existence side by side in the same culture; that is to say, it was necessary to reveal their coexistence in a given stock culture, without having recourse to dilution into plain broth.

*Experiment 3. Difference in Appearance of Colonies of the Granular and Diffuse Growing Microbes.*—A stock serum agar slant of Strain R 15 was subcultured into 5 per cent rabbit serum broth. The culture after 6 hours showed diffuse growth. It was diluted to  $10^{-8}$  cc. in plain broth, and subinoculations were made as before into parallel series of plain and serum broth tubes. The serum broth subculture showed diffuse growth in dilutions  $10^{-8}$  to  $10^{-2}$  cc. The plain broth parallel subcultures grew in dilutions up to  $10^{-6}$  cc., but not in dilutions  $10^{-7}$  or  $10^{-8}$  cc. All the tubes of this series were granular in their growth character.

The serum broth subculture,  $10^{-8}$  cc. (diffuse), and plain broth subculture,  $10^{-6}$  cc. (granular), were now transferred into 5 per cent serum broth. The transplant from the first grew diffusely and was designated D. That from the second sedimented rapidly; it was designated G. Cultures D and G were again carried over to serum broth. After 6 hours incubation at  $37^{\circ}\text{C}$ ., each tube was diluted in plain broth in the usual manner to  $10^{-8}$  cc. of the original. Tube G was thoroughly shaken to break up the clumps before dilution.

Dilutions  $10^{-7}$  and  $10^{-8}$  cc. of Cultures D and G were plated in 5 per cent serum agar. The surface colonies resulting from Inoculation D were strikingly different from those of Inoculation G. The former were whitish, with rather opaque, glistening centers, fading into translucent outer zones. Their borders were regular. They exhibited marked fluorescence, both by daylight and by artificial light. The surface colonies of Type G were somewhat smaller, translucent, and bluish in color, had irregular serrated edges, and showed little or no fluorescence.

Colonies of the first type, D, fished to 5 per cent serum broth, yielded diffuse growth. Those of Type G gave rise in every instance to granular, rapidly sedimenting cultures.

The fact that the difference in appearance of colonies accompanied the difference in growth character in fluid medium was utilized at once as a means of detecting whether Types G and D exist side by side in strains which have never been carried into plain broth. It will be seen by reference to Table I that the Type G organisms in the original culture are present in smaller numbers than those of Type D. Since, ordinarily, Type G grows in plain broth subcultures from dilutions not higher than  $10^{-4}$  cc., it will be evident that there is no hope of detecting Type G colonies by plating out high dilutions of serum broth cultures. Consequently, the demonstration of coexistence had to be sought by another method.

It was observed that when stock serum agar cultures of the various strains were planted in serum broth the resulting growth was diffuse, but that after 8 to 10 hours incubation there was always a more or less abundant sediment at the bottom of the tube. It was assumed that if the granular organisms were present with the diffuse variety, it would be logical to find them in high concentration in the sediment. This assumption was tested in Experiment 4.

*Experiment 4. Demonstration of the Coexistence of Types G and D by Plating the Supernatant Fluids and Sediments of Serum Broth Cultures.*—A stock 10 per cent serum agar slant of Strain R 22 was transferred to a tube of 5 per cent rabbit serum broth. After incubation at 37°C. for 24 hours, the tube contained a uniformly turbid supernatant fluid, and in addition a distinct sediment. The supernatant fluid was carefully drawn off by a Pasteur bulb pipette, care being taken not to disturb the sediment. The supernatant fluid was placed in a sterile tube, Tube A.

The sediment was now thoroughly shaken up in 10 cc. of sterile broth—Tube B. Tubes A and B were streaked upon plain and serum agar plates, which were then incubated for 24 hours. The result is summarized in Table II.

The two different type colonies were now fished from the serum agar plates to serum broth. Subcultures from the translucent, non-fluorescent type invariably yielded granular strains. Those from the opaque, fluorescing colonies gave rise to an invariably diffuse growth. Experiment 4 demonstrates clearly that Type G and D microbes

coexist in the stock serum broth tubes. It shows that Type G organisms, usually in the minority, can be easily demonstrated by the separation of the supernatant fluid and the sediment of a serum broth culture, and the streaking of the latter. It must be pointed out in this place that this method of procedure is only necessary when Type D greatly predominates. It has been found that when cultures of the rabbit septicemia bacillus are infrequently transplanted, the more saprophytic Type G tends to gain the upper hand. Under these conditions simple streaking out of a thoroughly shaken culture suffices to place both varieties in evidence.

TABLE II.  
*Dissociation of Types G and D by Sedimentation.*

Tube.	Material.	5 per cent serum agar plate.	Plain agar plate.
A	Supernatant fluid of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type D (fluorescent) preponderates.	Scanty growth; both types present, but Type D preponderates.
B	Sediment of 24 hr. serum broth culture, Strain R 22.	Good growth; both types present. Type G (non-fluorescent) largely preponderates.	Very scanty growth; almost entirely non-fluorescent Type G.

Up to the present three differential characteristics of Types D and G have been discussed. (1) Type G is able to grow much more easily than Type D in plain broth; that is to say, much larger amounts of Type D than of Type G are necessary to cause multiplication in this medium. (2) Type G grows in minute granules in liquid media, these particles sedimenting rapidly and leaving the supernatant fluid clear. Type D, on the other hand, grows diffusely, both in serum and in plain broth. (3) The colonies produced by Microbe D are distinctly different from those of Type G.

Careful study has revealed no difference in morphology or tinctorial reaction between Types G and D. Their fermentation reactions have proved to be identical. The question arises whether other differences exist.

Since Microbe G grows with distinctly greater ease in plain broth, it is easy to imagine that this fact might represent an adaptation of the organism, ordinarily rather delicately adjusted to the parasitic state, to a saprophytic existence; that is to say, Type G, which is more saprophytic, might exhibit a corresponding loss in parasitic activity, and hence might be of decidedly less virulence for rabbits than the corresponding Type D.

This hypothesis is found to be vindicated in a striking manner in Experiment 5.

TABLE III.  
*Comparative Virulence of Types G and D, Strain R 15.*

Type.	No. of passages after dissociation.	Weight of rabbit.	Age of culture.	Amount injected intra-pleurally.	Result.
		gm.	hrs.	cc.	
D	Serum broth third passage.	550	6	$10^{-7}$	Negative.
		550		$10^{-6}$	Died in 42 hrs.*
		550		$10^{-5}$	Died in 42 hrs.*
G	Plain broth first, serum broth second passage.	550	6	$10^{-7}$	Negative.
		550		$10^{-5}$	"
		550		$10^{-3}$	"

\* Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure culture of Type D from heart's blood.

Plate of dilution  $10^{-8}$  cc., Type G, 32 colonies.

Plate of dilution  $10^{-8}$  cc., Type D, 21 colonies.

*Experiment 5. Comparative Virulence of Types D and G.*—Microbe D was obtained by transplanting a diffuse growing serum broth subculture from dilution  $10^{-8}$  cc. of a 6 hour serum broth culture of Stock Strain R 15. Third passage in serum broth. Microbe G was obtained by transplanting a granular plain broth subculture from dilution  $10^{-6}$  cc. of the same serum broth culture of Strain R 15. Passage in serum broth exactly parallel to that of Microbe D.

Serum broth cultures of each variety were incubated for 16 hours and seeded in 0.05 cc. amounts into tubes of 5 per cent rabbit serum broth. Incubation for 6 hours at  $37^{\circ}\text{C}$ . Dilution in plain broth to  $10^{-8}$  cc., care being taken to disintegrate thoroughly the granules of Culture G. Culture D was then injected intrapleurally, in doses of  $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  cc., into rabbits of 550 gm. weight.

Culture G was injected by the same route into rabbits of the same weight in doses of  $10^{-7}$ ,  $10^{-5}$ , and  $10^{-3}$  cc. Dilutions of  $10^{-8}$  cc. of each culture were plated in 5 per cent serum agar. The result, which was most striking, is recorded in Table III.

Repetition of this experiment with the same and with other strains yielded constantly a like result. In a word, Microbe G, recently dissociated from a stock strain, shows itself to be distinctly less virulent than its companion Type D of the same strain. All experiments of this type were made with organisms dissociated from the

TABLE IV.

*Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.*

Type.	Strain.	No. of passages in serum broth.	Age of culture.	Amount injected intra-pleurally.	Result.
G	R 22	7	6 hrs., 1,100,000,000 colonies per cc.	cc.	
				$10^{-4}$	Negative.
				$10^{-3}$	"
				$10^{-2}$	"
D	R 22	7	6 hrs., 1,140,000,000 colonies per cc.	$10^{-1}$	"
				$10^{-6}$	"
				$10^{-5}$	Died in 16 days.*
				$10^{-4}$	Died in 4 days.*
				$10^{-3}$	Died in 39 hrs.*

\* Autopsies in all cases typical. Pure Type D recovered from heart's blood of each animal.

same strain, at the same time, and transplanted under rigidly parallel conditions. The results in Table III are by no means the most striking obtained, since young rabbits frequently resist 0.1 cc. of a 6 hour culture of Type G, while parallel Type D cultures are often fatal in dose of  $10^{-7}$  cc. and never in less than  $10^{-4}$  cc.

It might be objected in the experiment just described that the Type G organisms had lost their virulence by reason of their transplantation, for one passage, in plain broth. The method of dissociation by sedimentation described in Experiment 4 makes it easy to



examine into the validity of such an objection. This technique was therefore applied to Strain R 22, and the virulence of the resulting Type G and D varieties tested. In this case Strain G had received no passage through plain broth.

*Experiment 6. Virulence of Types D and G, Strain R 22, Dissociated by the Sedimentation Method.*—In order to insure the purity of each type, three successive platings in serum agar, with alternate transfers into serum broth, were made, after the primary separation by sedimentation. After the third plating, Microbes D and G were carried for three passages, daily transfer, in serum broth. Each was then subinoculated from a 12 hour serum broth culture into a tube of 5 per cent rabbit serum broth. Incubation at 37°C. for 6 hours; dilution, as usual, to  $10^{-7}$  cc. of the original culture. Type G was then injected into 1,500 gm. rabbits, intrapleurally, in doses of  $10^{-4}$ ,  $10^{-3}$ ,  $10^{-2}$ , and  $10^{-1}$  cc. Type D was injected by the same route into rabbits of the same size, in doses of  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  cc. The result is given in Table IV.

It must be stated here that the infection in rabbits of large size is identical in nature with that of young animals, the sole difference being in the time of death.

Experiments 5 and 6 show clearly that Microbe G is distinctly less virulent than Microbe D. It was natural to ask whether Microbe G could produce typical infections when injected in overwhelming quantities. It was found that adult rabbits tolerated doses of 0.5 cc. of whole serum broth culture. Young rabbits were for this reason selected for the experiment.

*Experiment 7. Effect on Young Rabbits of Massive Doses of Type G.*—A series of 600 gm. rabbits was injected intrapleurally with 3, 2, 1, 0.5, and 0.1 cc. of a 6 hour culture of Strain R 15, Type G. The rabbit injected with 3 cc. succumbed in 5 days. Those that received 2 and 0.5 cc. died in 32 and 24 hours, while doses of 1 and 0.1 cc. produced no lethal effect. It is important that the organisms recovered at necropsy showed a granular growth in serum broth, and when streaked upon serum agar plates gave rise to typical Microbe G translucent colonies.

The pathologic effects produced by Microbe G as compared with those produced by the more virulent Microbe D will be dwelt upon in a later communication in which the possibility of raising the virulence of Type G will be discussed. It is enough to say that this microbe retains its character of growth after one passage through the animal body.

It is frequently remarked that the virulence of many organisms is lost more quickly by cultivation in ordinary media than in media enriched with serum or blood. Observations have shown that with Type D (virulent) of various strains of the organisms under consideration, the characteristic diffuse growth is retained despite many passages in plain broth. Since it has been noted that the characters of diffuse growth and virulence in this instance accompany one another, it was considered important to discover whether plain broth passage would depress the virulence of Microbe D, while the diffuse growth character remained. Experiment 8 supplies an unequivocal answer to this question.

*Experiment 8. Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Plain and Serum Broth.*—Microbes D and G were dissociated from Stock Strain R 15 by the first of the methods described above; *i.e.*, by dilution and parallel subculture in plain and serum broth. They were plated three successive times in serum agar, isolated colonies being fished to serum broth each time. This insured as nearly as possible that the strains arose from a single organism. Microbe D was then carried in parallel passage through twenty-five daily transplantations in plain and in serum broth. From time to time the cultures were streaked on serum agar plates. With both the plain and the serum broth tubes organisms of Type D only were invariably found. What is more, the plain broth strain retained perfectly its characteristic of diffuse growth. The plain broth passage strain is designated as D-Pl, that of the serum broth passage as D-S.

Microbe G, dissociated at the same time, was subjected to a similar number of passages in serum broth. The virulence of all three of the strains was now tested. 6 hour serum broth cultures of Microbes D-S, D-Pl, and G-S were diluted in plain broth to  $10^{-7}$  cc. of the original cultures. The injection of the test animals was carried out immediately after the dilution of each culture. At the end of the injections plates were in each instance made in 5 per cent serum agar. The result is summarized in Table V.

Table V shows very clearly that plain broth passage has little or no effect upon the virulence of Microbe D. On the other hand, Microbe G characteristically fails to kill in 0.1 cc. Up to the present time we have been unable to discover Type D varieties possessing low virulence in any of our strains. What is more, it is a remarkable fact that Strains R 19 and R 21, which so far have shown no evidence of organisms of Type G, are by far the most invasive of any of the

strains studied. This experiment, together with other observations described above suggest that the mechanism of attenuation may be a selection; that is, an overgrowth of the virulent Type D by the less virulent Type G. This hypothesis is amenable to experimental proof and will be discussed fully in a later communication.

TABLE V.

*Comparative Virulence of Type D, Strain R 15, after Twenty-Five Parallel Passages in Serum and Plain Broth.\**

Type.	Strain.	No. of passages.	Weight of rabbit.	Age of culture.	Amount injected intrapleurally.	Result.
			gm.	hrs.	cc.	
D	R 15	Serum broth, twenty-five passages.	700	6	$10^{-6}$	Died in 24 hrs.†
			725		$10^{-5}$	" " 36 " †
			650		$10^{-4}$	" " 42 " †
			675		$10^{-3}$	" " 38 " †
D	R 15	Plain broth, twenty-five passages.	700	6	$10^{-6}$	" " 36 " †
			750		$10^{-6}$	" " 24 " †
			750		$10^{-4}$	Negative.‡
			700		$10^{-3}$	Died in 31 hrs.†
G	R 15	Serum broth, twenty-five passages.	700	6	$10^{-4}$	Negative.
			700		$10^{-3}$	"
			650		$10^{-2}$	"
			675		$10^{-1}$	"

\* Microbe G-S 25, control.

† Autopsy typical; fibrinopurulent pleuritis, pericarditis, and bronchopneumonia. Pure cultures of Type D from heart's blood.

‡ Probably technical error in inoculation.

Number of colonies in  $10^{-7}$  cc.: Microbe D-S, 30; Microbe D-Pl, 53; Microbe G-S, 10.

### *Immunologic Relations of Types G and D.*

Microbes G and D in all instances so far studied differ in growth character in fluid media, in colony formation, and in virulence. It is logical to inquire into their immunologic relations. The first experiment consisted in determining the degree of resistance offered by animals which had survived injection with Microbe G to injections of multiple lethal doses of the virulent Type D.

*Experiment 9. Resistance of Animals Which Had Survived Injection with Type G to Multiple Lethal Doses of Type D.*—Rabbits 1 and 2, weighing 1,500 gm. each, withstood with no visible effect 0.1 and 0.5 cc. of Type G, Strain R 15, injected intrapleurally. 14 days later they were injected by the same route with  $10^{-4}$  cc. of a 6 hour serum broth culture of the virulent Type D, Strain R 15. Immediately afterward, controls of the same weight received doses of  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  cc. of the same culture. The result is summarized in Table VI.

As seen from Table VI, Rabbits 1 and 2 were resistant to at least 100 lethal doses of the virulent Strain D. It is of interest that such solid immunity should be conferred by a single injection of the less virulent Strain G. Other experiments gave the same result. It

TABLE VI.

*Resistance of Animals Which Had Survived Injection with Type G, Strain R 15, to Multiple Lethal Doses of Type D, Strain R 15.*

Rabbit No.	Amount of Type G injected intrapleurally 14 days previously.	Weight of rabbit.	Amount injected for test of Type D.	Result.
	cc.	gm.	cc.	
1	0.1	1,500	$10^{-4}$	Negative.
2	0.5	1,500	$10^{-4}$	"
3		1,450	$10^{-6}$	Died in 9 days.*
4		1,500	$10^{-5}$	" " 4 " *
5		1,475	$10^{-4}$	" " 4 " *

\* Autopsy typical. Pure Type D recovered from heart's blood.

Number of colonies on serum agar plate of dilution  $10^{-8}$  cc., 16.

must be remarked that no experiments have been instituted that would indicate the comparative immunizing efficacy of the virulent Type D and the less virulent Type G.

#### *Agglutination Reactions of Types G and D.*

The vaccinating power of Type G against the virulent Type D has been clearly demonstrated in Experiment 9. The results of agglutination studies bear out the community of antigenic quality suggested by the vaccination experiments. Stable suspensions of Microbe G (granular) may be prepared by washing the organisms from 5 per cent serum agar slants four times in distilled water, and

finally suspending them in this medium. 0.1 per cent formaldehyde was added to all suspensions.

The flocculation of both Types G and D by immune sera is very slow and is much more clear-cut at 55° than at 37°C. The necessity of incubation at 55° is especially marked with Type D. This fact is, indeed, of differential value, since Type G suspensions agglutinate very well when placed in contact with immune serum at 37°C. for 16 hours. Parallel tests made on Microbe D at the same temperature do not give satisfactory results.

Serum prepared by three intravenous injections of Microbe D into rabbits agglutinates Types D and G in titer of 1:2,000 after incubation at 55° for 16 hours. A serum resulting from similar injection of Microbe G is considerably less active, agglutinating Type G in 1:1,000 and Type D in 1:200. In some instances Microbe D agglutinated in dilution of 1:50 but not in higher dilutions. To sum up, the antigenic power, as far as the production of agglutinin is concerned, appears to be decidedly stronger in the case of Type D than of Type G.

Absorption tests clearly indicate the community of antigenic character of the two types. Serum > Type D, Strain R 15, agglutinated Microbes D and G in titer of 1:1,500. After 2 hours contact at 55° with a suspension of Microbe D, the titer for Type G had fallen to 1:200, for Type D to 1:80. The control serum, after 2 hours at 55° without the suspension, showed the original titer, 1:1,500. The same serum, placed for a similar length of time in contact with Microbe G, dropped in titer from 1:1,500 to 1:40 for Types D and G. Various observations make it apparent that Microbe G, in addition to being more easily flocculable, also has greater binding power for the agglutinating principle than has Microbe D. From the foregoing results it is apparent that there is no qualitative difference in the antigenic nature of Types G and D. In a later communication these agglutination reactions will be dealt with more fully, and the acid flocculability of the two types will be discussed. The acid agglutination points of Types D and G are distinctly different. They are of the nature of physical constants, and hence present a valuable differential criterion.

## SUMMARY AND CONCLUSIONS.

Two types of organism have been shown to exist in cultures of the bacillus of rabbit septicemia, recently isolated from spontaneous infections.

One, Microbe D, grows diffusely in serum and plain broth, forms rather opaque, fluorescing colonies on serum agar, and is highly virulent for rabbits. These characters are retained throughout many passages in serum or plain broth.

The other type, Microbe G, flocculates rapidly in fluid media, forms translucent, bluish colonies with little fluorescence, and exhibits extremely low virulence for rabbits. Like Microbe D, its distinguishing characters persist throughout many passages in artificial media.

Two methods for the dissociation of these varieties from the parent culture have been described.

The two types are morphologically indistinguishable and possess identical fermentation reactions.

Rabbits surviving inoculation with Type G are resistant to multiple lethal doses of Type D. The agglutination reactions bear out this suggestion of the antigenic identity of the varieties. Community of antigenic character is rendered certain by the results of absorption reactions.

Microbe D, in contact with immune serum, flocculates well at 55°, but poorly or not at all at 37°C. Microbe G, on the other hand, agglutinates easily at both temperatures.

Microbe D, after being carried through twenty-five passages in serum and in plain broth, retains perfectly its characteristics of diffuse growth and of virulence, in the plain as well as in the serum broth.

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STUDIES ON DECREASING THE REACTION OF NORMAL  
SKIN TO DESTRUCTIVE DOSES OF X-RAYS BY  
PHARMACOLOGICAL MEANS AND ON  
THE MECHANISM INVOLVED.\*

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PLATES 100 TO 102.

(Received for publication, March 18, 1921.)

In an earlier investigation evidence was presented to show that an organism sensitized by a foreign protein could locally autoinoculate itself with the same protein when certain conditions were fulfilled.<sup>1</sup> As this mechanism would serve to explain a number of abnormal reactions of hitherto cryptogenetic origin, it was desirable to advance still more proof. For this reason work was undertaken in which x-rays were the local irritating agent which caused the autoinoculation. During the preliminary stages of this research, however, an impression gradually developed that a certain group of rabbits seemed to show an increased resistance to doses of ordinarily destructive x-rays. Since this would be of considerable theoretical as well as practical value, if true, we abandoned our original object temporarily in order to study this point. The result demonstrated the correctness of the impression that the skin after the systemic incorporation of serum could be rendered resistant to doses of x-rays which are lethal to the tissues of non-prepared animals.

\*A preliminary statement was recently published (Auer, J., and Witherbee, W. D., *J. Am. Med. Assn.*, 1921, lxxvi, 301).

<sup>1</sup> Auer, J., *J. Exp. Med.*, 1920, xxxii, 427.

*Method.*

An interruptorless, 10 kilowatt, 220 direct current machine with a medium focus Coolidge tube was used. After preliminary trials 30 skin units (Witherbee-Remer formula)<sup>2</sup> were chosen as the standard test dose of x-rays. This was produced by a 3 inch spark-gap, 10 milliampere current, 6 inch distance from target, and 20 minute exposure. All these factors were constantly controlled throughout the period of treatment of all the animals. No filter was employed, except that a disc of ordinary filing card was placed between the tube and the skin surface in order to reduce the heat effect.

Rabbits only were used. The area x-rayed was always 4 sq. cm. of the upper half of the right ear, the central artery of the ear passing through the middle of this space. The rest of the ear and body was protected by a sheathing of lead. Shifting of the x-rayed area, due to movements of the animal, was minimized by a simple device. The right ear was turned forward, smoothed out upon a small board and a strip of plaster fixed the tip of the ear to the board and the board to the box. A mask of sheet lead provided with an opening of 2 by 2 cm. was carefully placed in position on the right ear and held there by a strip of plaster. After covering the rest of the head and the entire box with lead sheeting, the animal was ready for treatment. Great care was exercised to prevent a circular constriction of the neck. This procedure was quite successful, though a moderate lateral shifting of the ear occurred in some instances.

It should be emphasized that the site chosen for x-raying offers a number of advantages: the ear is always easily available for inspection with no discomfort to the animal; two skin surfaces are affected by the x-rays, the dorsal on entry and the internal surface on exit of the x-rays; no serious systemic effects are to be feared even after massive doses because the x-raying is entirely localized to a comparatively small area; the ear of the rabbit is richly vascularized, and possesses a number of direct arteriovenous anastomoses<sup>3</sup> which guarantee an especially efficient collateral circulation.

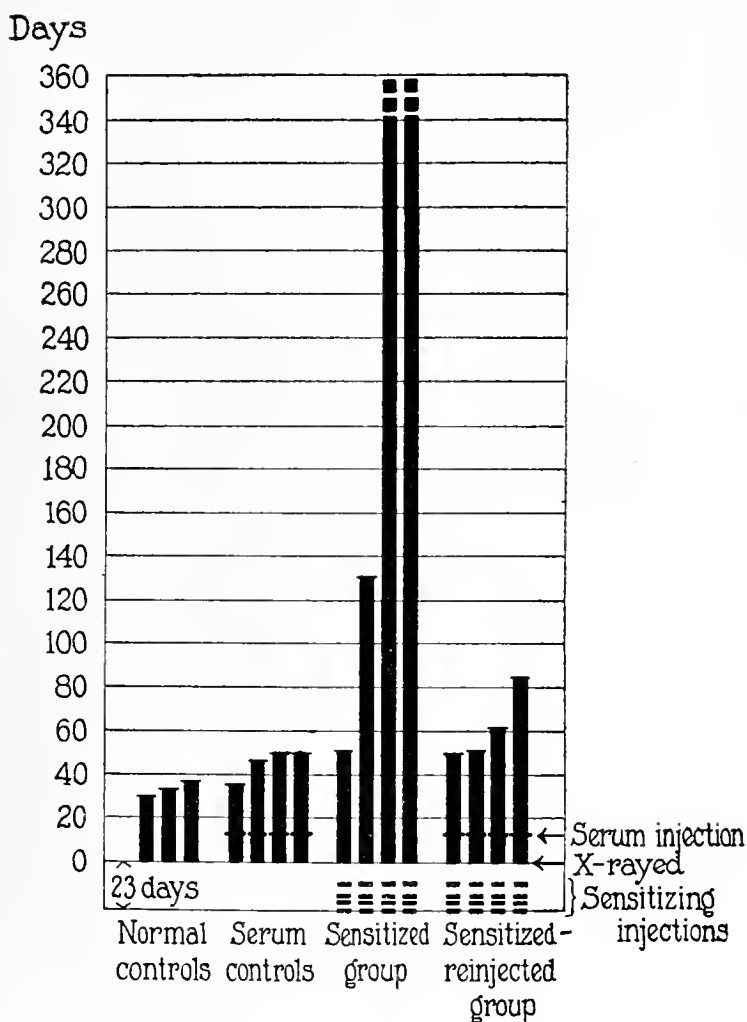
All animals except the normal controls were x-rayed on the same day, but the members of no group were x-rayed in succession. The procedure was to take one animal from each group in rotation until all animals had been exposed to the x-rays.

After the rabbits had been x-rayed they were examined at 2 to 4 day intervals or daily when necessary, for a period of over 300 days, at which time the evidence was deemed sufficient to terminate this aspect of the work.

The end-point of the reaction was the appearance of a spot of dry gangrene in the x-rayed area, with subsequent fenestration. The number of days which elapsed between the time of x-raying and the appearance of dry gangrene, or the

<sup>2</sup> Witherbee, W. D., and Remer, J., *Arch. Dermatol. and Syphilol.*, 1920, i, 558; *Am. J. Roentgenol.*, 1920, vii, 485.

<sup>3</sup> Berlinerblau, F., *Arch. Anat. u. Physiol.*, 1875, 177.

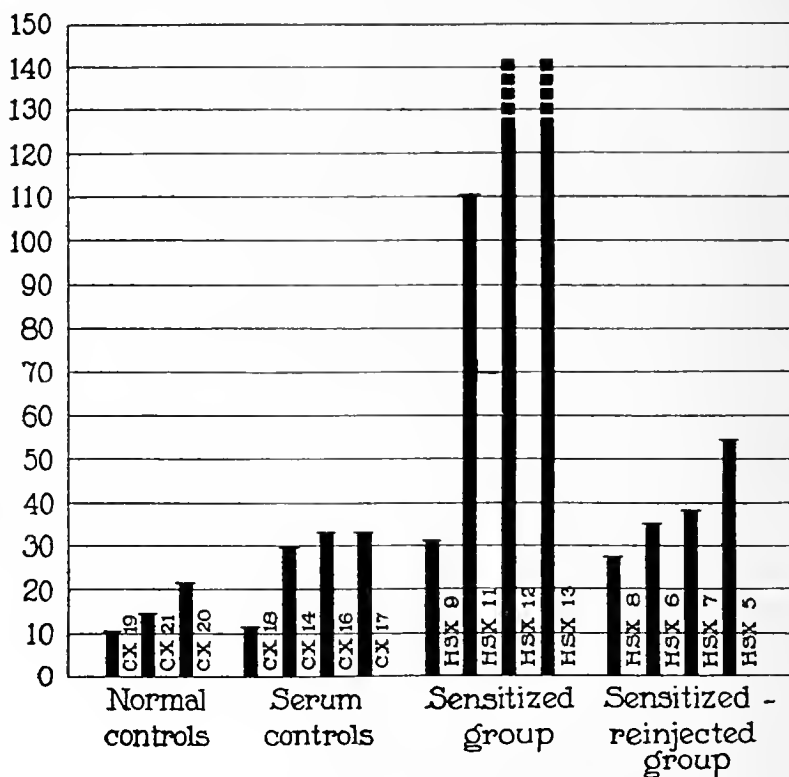


TEXT-FIG. 1. Duration of the reaction from the day of x-ray treatment to the appearance of perforating gangrene.

duration of the inflammatory process up to gangrene, was then plotted. Text-figs. 1 and 2 bring out well the striking difference between the various groups.

The type of rabbit employed, their feeding, care, method of injection, etc., have been described in an earlier paper.<sup>1</sup>

Days



TEXT-FIG. 2. Duration of the reaction from the onset of the first exudate to the appearance of perforating gangrene.

The experimental animal material was composed of four groups of rabbits: (1) normal controls, (2) serum controls, (3) sensitized group, and (4) sensitized-reinjected group.

The normal controls were normal, untreated rabbits in which various doses of x-rays were tested as described. Both ears were utilized at different times. There were six rabbits in this group; in three the standard dose was used.

The serum controls, five in number, were normal rabbits which received a single injection of 10 cc. of horse serum<sup>4</sup> intraperitoneally, 13 days after the ear had been x-rayed.

The sensitized group of rabbits, five in number, was sensitized by two subcutaneous and two intramuscular injections of 1 cc. of horse serum each, at 3 to 4 day intervals. 10 days after the last sensitizing dose the right ears were x-rayed locally with the standard dose.

The sensitized-reinjected group, five rabbits, was prepared exactly as has been described for the sensitized group, but 13 days after x-ray treatment this group was reinjected intraperitoneally with 10 cc. of horse serum. This group was thus subjected to an anaphylactic reaction 23 days after the last sensitizing dose of serum. Text-fig. 1 shows the relation of the groups and the various procedures.

During the early stages of the work one rabbit in each of the last three groups died without obvious lesions. These groups therefore now consisted of four rabbits each.

#### RESULTS.

Before presenting the results in detail we shall first give the main outstanding facts of this work.

The normal control rabbits developed dry gangrene and fenestration in the x-rayed area in 30, 33, and 37 days respectively.

The serum control rabbits showed dry gangrene and fenestration in the x-rayed ears after 36, 47, 50, and 50 days respectively (Figs. 1 and 2).

The sensitized-reinjected group exhibited the same lesions after 50, 52, 62, and 85 days respectively (Figs. 5 and 6).

The sensitized group, however, responded quite differently on the whole. Only one rabbit showed gangrene and fenestration of the ear in 46 days. A second rabbit developed the same lesion, but only after 131 days. The two remaining rabbits have developed no gangrene or fenestration even after the lapse of more than 340 days (Text-figs. 1 and 2 and Figs. 3 and 4).

These data demonstrate clearly that rabbits previously sensitized by the parenteral injection of horse serum acquire a remarkably increased resistance in the majority of instances to doses of x-rays which are lethal to the tissues of normal control rabbits or serum control rabbits.

<sup>4</sup>The horse serum was kindly furnished by Dr. W. H. Park and Dr. E. J. Banzhaf of the Department of Health of the City of New York.

The results also show convincingly that the protection which serum sensitization previous to x-ray treatment confers is largely abolished when the sensitized and x-rayed animals are subjected to a general anaphylactic reaction (Text-figs. 1 and 2, sensitized-reinjected group).

The main objective details of the investigation are as follows: Within 24 hours after x-ray treatment two to three rabbits out of each group of five showed a slight pinkness of the x-rayed area which disappeared within 2 to 3 days. This pinkness is probably due to a heat effect from the Coolidge tube. Within 4 days the hair of the x-rayed area began to loosen, though there was considerable variation. Thus for example on the 11th day after x-ray treatment some ears showed bald patches, while in others the hair was still firmly fixed. This variation bore no relation apparently to the experimental group. Pigmentation of the x-rayed area was noticeable in 2 to 4 days after x-ray treatment and varied with the different animals. In some it became very marked, while in others the pigmentation was always slight. The degree of pigmentation bore no definite relation to the group to which the animal belonged. A slight thickening of the x-rayed area, without any obvious vascular congestion, was first noticed 9 days after x-ray treatment; it occurred in one to three rabbits of each group. After 11 days these rabbits showed, in addition to the thickening, a slight but definite congestion of the x-rayed area. 13 days after x-ray treatment all rabbits, except two members of the sensitized-reinjected group, showed a definite though slight congestion with slight thickening of the x-rayed area. It should be noted that no rabbit had yet been reinjected with serum at the time of this examination.

The congestion of the x-rayed area increased slowly, but not at an equal rate in all the groups. Thus, 16 days after x-ray treatment the serum control rabbits still exhibited only a slight congestion of the x-rayed area, while the sensitized group showed a moderate to marked congestion, and the sensitized-reinjected animals a fair to moderate congestion. Associated with the increased congestion there was also a slight increase in the thickness of the x-rayed patch.

After 18 to 25 days generally the x-rayed area developed an exudate on both surfaces which dried into crusts. The healing of this first inflammation was usually complete within 28 to 36 days and the x-rayed areas now appeared like healed superficial wounds. The x-rayed area was absolutely bald and practically free of crusts; the skin was thin, whitish, glistening, and easily crinkled into thin folds; the blood supply was good, though many rabbits showed pearly white spots in the x-rayed areas; there was no gangrene.

This termination of what we shall call the first inflammation did not take place in all rabbits, but occurred in a majority of the serum controls, the sensitized group, and the sensitized-reinjected group (Table I). In the normal controls (three rabbits) this first inflammation with crusts did not clear up but passed at once to a complete perforating gangrene. The same fact was also observed once in the serum control group (No. CX 18) and once in the sensitized-reinjected group (No. HSX 8) (Table I).

TABLE I.

Group.	Series No.	First inflammation.		Interval between first and second inflammation.	Second inflammation.		Delayed second inflammation.	Length of observation.
		Day of onset of exudate.	Day of healing.		Day of onset of exudate.	Day of appearance of gangrene.		
Normal controls.	CX 19	22nd	No healing before gangrene.	days				days
	CX 20	16th	" "	"	"	33rd	—	69
	CX 21	16th	" "	"	"	37th	—	69
Serum controls.						30th	—	69
	CX 14	18th	28th	8	36th	46th-49th	0	300+
	CX 16	18th	36th	6	42nd	49th-52nd	0	300+
	CX 17	18th	36th	13	49th	49th-52nd	0	300+
	CX 18	25th	No healing before gangrene.			36th	171st-195th	300+
Sensitized group.	HSX 9	15th	28th	14	42nd	46th	0	300+
	HSX 11	20th	28th	68	96th	128th-135th	0	300+
	HSX 12	20th	36th	At least 340.	0	0	0	300+
	HSX 13	23rd	28th	At least 340.	0	0	0	300+
Sensitized and reinjected group.	HSX 5	31st	42nd	10	52nd	82nd-89th	0	300+
	HSX 6	16th	36th	13	49th	49th-52nd	0	300+
	HSX 7	25th	49th	3	52nd	61st-64th	0	300+
	HSX 8	25th	No healing before gangrene.			52nd	0	300+

The figures represent the number of days after x-ray treatment unless otherwise stated. In all rabbits 4 sq. cm. of the right ear were x-rayed with 30 skin units.

The recovery from the first inflammation and the disappearance of the crusts were, however, not permanent in all the rabbits. After a period during which the x-rayed areas looked like healed or practically healed surface wounds, another exudate appeared unexpectedly on these x-rayed surfaces. This typical, second inflammation always led to a perforating gangrene (Table I). The interval elapsing between the end of the first inflammation and the onset of the second inflammation varied from 3 to 13 days in the serum controls and the sensitized-reinjected group. In the sensitized group, on the other hand, the interval before the second inflammation appeared was 14 days in one rabbit, 68 days in a second, and in the two remaining rabbits no second inflammation leading to gangrene has developed after more than 340 days (Table I and Text-fig. 1).

The character of the second inflammation occurring in the x-rayed area was interesting. A fairly marked redness and swelling of the x-rayed tissue preceding gangrene were observed only once (No. HSX 11), and in all the other rabbits in which fenestration of the ear developed, the inflammatory signs were a moderate pinkness of the x-rayed tissue with no definite swelling or heat; the most noticeable feature was the appearance of a sticky exudate, often on both ear surfaces. This exudate apparently was poured out at various rates, for the subsequent crusts exhibited a definite lamination<sup>5</sup> in many instances.

The difference between the inflammatory reaction of the x-rayed tissue and the normal surrounding tissue was well illustrated in four animals, one in each group—Nos. CX 21, CX 14, HSX 11, and HSX 8 (Table I). In these rabbits an inflammation of the right ear set in, perhaps due to scratching. This inflammation was most marked around the periphery of the x-rayed area, especially at the upper and lower borders. In the untreated part the tissues were red, swollen, hot, and in two rabbits (Nos. HSX 11 and HSX 8) a tongue of edema ran from the lower border of the x-rayed area towards the base of the ear; the blood vessels of the untreated part of the ear were markedly dilated. The inflamed tissue, however, stopped sharply at the x-rayed area, and this latter tissue, while more or less pink in all, stood out in striking contrast to the inflamed surroundings, which thus framed the comparatively pallid x-rayed area. The central artery, turgid with blood above and below the x-rayed area, was a mere red thread within this space. There was no appreciable thickening of the x-rayed area on palpation except in No. HSX 11, in which a well marked edema of the lower portion set in. The second inflammation of the x-rayed area was therefore in general of a definite subacute character, while the first inflammation resembled a mild, acute inflammation.

Another striking difference between the x-rayed and normal tissues was observed in the development of exudate and crusts in the four rabbits mentioned above. The marked inflammation of the normal ear tissue did not lead to exudate and crust formation, while thick crusts often developed in the x-rayed areas.

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<sup>5</sup> This lamination was observed in the secondary exudate; no notes were made on the structure of the crusts in the primary exudate.



The chief sign which heralded the onset of a perforating gangrene was the appearance of a small, brown-black, slightly sunken spot on the internal skin surface of the x-rayed area; occasionally an exceptionally thick crust was the first sign. In the last three groups the sunken, discolored spot was noted seven times in the ten rabbits in which fenestration took place. The appearance of this discoloration, however, did not invariably foretell the onset of a perforating gangrene. Thus No. HSX 13 exhibited a brown discoloration of the internal surface with slight, thin crust formation 64 days after x-ray treatment. This lesion did not progress, but was practically healed on the 86th day. It will be remembered that this rabbit belongs to the sensitized group and showed no gangrene of the x-rayed area within more than 340 days after x-ray treatment (Table I and Text-fig. 1).

The initial point where a perforating gangrene developed was small when first observed, at times not more than 1 to 2 mm. in diameter. This dry spot then increased in size, first rapidly, then slowly until an equilibrium was established between the destructive and reparative factors. Several times two spots of gangrene developed, one on each side of and close to the central artery of the ear. These two spots always fused sooner or later, but the gangrenous process was always more rapid away from the artery than towards it, though finally the intervening section of the artery also dried up.

The amount of tissue lost by gangrene was never equal to the entire area x-rayed; in only two instances did the loss exceed 50 per cent. The gangrenous process began near the center of the area x-rayed and then progressed towards the borders. This spread was usually greater in the lateral direction than towards the root or apex of the ear. In the serum control group the loss of tissue varied between 80 and 130 sq. mm.;<sup>6</sup> in the sensitized group (two rabbits) between 117 and 224 sq. mm.; and in the sensitized-reinjected group the loss fluctuated between 70 and 210 sq. mm.

The measurements given are only rough approximations of the losses, and no effort was made to determine the true areas of the more or less irregular fenestrations. We believe, however, that the figures convey a just impression.

In addition to the second, subacute type of inflammation leading to gangrene, which has been described, a delayed, second form of subacute inflammation also leading to gangrene may be distinguished. This form was observed only once; it occurred in Rabbit CX 18 of the serum control group (Table I). In this rabbit the first inflammation beginning 25 days after x-ray treatment led at once to a perforating gangrene 36 days after treatment. A similar acceleration of the process took place in No. HSX 8 of the sensitized-reinjected group and in all the

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<sup>6</sup> No measurements were made in the last rabbit, No. CX 18, because the gangrene involved the border of the ear, due to a shift during x-ray treatment. Here also the loss was less than 50 per cent of the x-rayed area. The loss of tissue sustained by the normal controls was not measured, due to an oversight.

normal controls (Table I). In No. CX 18, however, a subacute inflammation leading again to gangrene developed 135 days after the first. During this interval of time the remainder of the x-rayed area did not exhibit any obvious differences from the x-rayed areas of other rabbits.

### *Final Changes in the X-Rayed Area.*

When the x-rayed areas of all the rabbits are examined some months after the last inflammation, all of them, including Rabbits HSX 12 and HSX 13 which never developed a perforating gangrene, show a number of changes in common. In all, the x-rayed area is hairless, the skin covering this area is smooth with perhaps a slight branny desquamation on the dorsal surface, and this skin wrinkles readily into thin folds. Occasionally, especially on the internal surface, numerous small, oval, yellowish brown thickenings of the outer skin are observable. These are less than 1 mm. in diameter and still less in thickness. They are seated in cup-shaped depressions of the skin, and probably represent keratoses.

The borders of the fenestrations generally show little or no thickening, but at or near the fenestration one or more red or reddish brown, slightly elevated papules are observable. These papules are formed by a number of dilated, small blood vessels. Occasionally, a slight hemorrhage proceeds from the angiectasias and the blood may burrow under the outer layers of the skin epithelium. These small masses of dilated blood vessels were also observed in Rabbit HSX 13 in which no perforating gangrene occurred; they were not seen in Rabbit HSX 12 of the same group.

In addition to these angiectasias the x-rayed areas show a number of tortuous blood vessels; often they are especially evident upon the internal surface. In the two rabbits of the sensitized group, Nos. HSX 12 and HSX 13, in which gangrene of the x-rayed area did not develop, these tortuous blood vessels are especially noticeable about the neighborhood of the central artery, where they form a delicate tracery of blood channels which are apparently superficial. The central artery itself in the x-rayed area of these two rabbits is narrow, slightly irregular in outline, and looks blurred in that portion of its course where the angiectasias are most marked.

The blood vessels of the healed, x-rayed areas do not react normally. In the normal rabbit the ear vessels respond by an initial blanching when the animal is sharply tapped, or a moderate struggle is induced; this blanching is followed by a marked vasodilatation if the original stimulus was sufficiently strong and if the room is not too cold. In the x-rayed areas of the experimental rabbits, however, this test causes at first some increase in the pallor, which later is replaced by only a slight dilatation of both arteries and veins. This striking difference in the vasomotor response of the x-rayed and untreated ears is well illustrated by Figs. 1 to 6, which were obtained by photographing the ears of two members of each group of rabbits during the stage of vasodilatation. It will be noticed that

both arteries and veins show a definite narrowing of caliber on entry of the x-rayed area, and that the original caliber is largely if not entirely regained when these vessels issue from the x-rayed area.

#### DISCUSSION.

From the experimental facts described above and summarized in the table and charts, it is clearly evident that the skin of rabbits under certain conditions may acquire a remarkably increased resistance to doses of x-rays which are surely destructive to control animals. These conditions are that the animal whose skin tolerance to x-rays is to be increased must be sensitized with horse serum and this sensitization must take place before the rabbit is exposed to the x-rays.

The evidence for these conclusions is summarized in Text-figs. 1 and 2. In these charts it is shown that the standard dose of 30 skin units of x-rays causes a perforating gangrene of the ear in normal controls within 37 days after x-ray treatment. The same dose of x-rays administered to the ear of rabbits previously sensitized with horse serum (sensitized group in the chart) was, however, remarkably weakened in its effect upon the tissues exposed to the x-rays. Two animals showed no gangrene at all during the period of examination (over 340 days); one exhibited a perforating gangrene after the lapse of 131 days, and only one member of the group of four rabbits reacted fairly like the normal controls by developing a perforating gangrene in 46 days. That sensitization must be present before the animal is exposed to the standard test dose of x-rays, if protection from the ordinarily destructive effects of this dose is desired, is shown by the serum control group. These rabbits were normal animals and were injected with horse serum for the first time, but this injection took place 13 days after exposure to the x-rays. In this group all rabbits developed fenestration of the ears subsequent to dry gangrene within 50 days after exposure to the x-rays. The serum injection after x-ray treatment therefore conferred no marked trace of protection to the x-rayed areas of the ears.

Additional evidence to establish this point, that sensitization previous to x-ray treatment confers a marked increase in resistance, is furnished by the behavior of the x-rayed area in Rabbit HSX 13 of the sensitized group. In this animal the x-rayed area a number of times exhibited some crust formation with moderate congestion of the surrounding vessels. In addition, the internal surface presented a brownish, sunken discoloration such as frequently preceded the appearance of a perforating gangrene in the x-rayed areas of the control rabbits. Yet healing was fairly prompt and no perforating gangrene resulted. The recuperative power of this x-rayed area, therefore, was greater than that existing in the x-rayed areas of the controls.

Another observation which points to the same conclusion is the inflammatory reaction which occurred previous to fenestration within a portion of the x-rayed area of a sensitized rabbit, No. HSX 11. This inflammatory reaction was accompanied by a fair degree of redness and swelling and was much more pronounced than that observed in the x-rayed area of any other rabbit, though it was considerably less than the inflammation which the same rabbit showed in the adjoining untreated portion of the ear. This increased inflammatory response can only be interpreted as an expression of a more vigorous state of this x-rayed area when compared to that of rabbits of other groups.

Another fact which seems clear is that the protection to x-rays which sensitization previous to x-ray treatment gives, is largely abolished if these animals are reinjected with serum after being x-rayed; in other words, if they are subjected to an anaphylactic reaction.

The evidence for this statement is summarized in Text-figs. 1 and 2. The sensitized and reinjected group, it will be seen, was treated exactly like the sensitized group with but one exception: 13 days after being x-rayed and 23 days after the last sensitizing dose of serum, this group was reinjected with horse serum, and in consequence a mild, general anaphylactic reaction resulted, from which all promptly recovered. Nevertheless, the further course of the experiment showed that these reinjected rabbits had largely lost the protection which mere sensitization gives (see the sensitized group, Text-figs. 1 and 2), and dry gangrene with fenestration took place in due time. That some protection had still remained, however, is indicated by the fact that the interval between x-ray treatment and gangrene is appreciably longer in two animals (62 and 85 days respectively) than in any of the controls (see also Text-fig. 2).

The increased resistance of skin-covered tissues to unfiltered x-rays which results from previous sensitization with an undenatured protein may be roughly estimated from our data. In preliminary experiments we tested the effects of 15, 18, and  $22\frac{2}{3}$  skin units of x-rays on areas of rabbits' ears 4 sq. cm. in size. With  $22\frac{2}{3}$  units, perforating gangrene occurred in the two rabbits tested within 37 to 43 days. With 18 skin units, perforating gangrene took place 58 days after x-ray treatment in one rabbit, and incomplete gangrene in two others after 72 days, when observations were discontinued. With 15 skin units complete gangrene occurred in one rabbit after 91 days, incomplete gangrene in a second after 91 days, and no gangrene at all in a third animal after the same interval, when, unfortunately, all these rabbits were discarded.

From these incomplete data we may nevertheless conclude that sensitization with horse serum previous to x-ray treatment can reduce, at least in some animals, the destructive value of 30 skin units of x-rays to a level of 15 to 18 skin units.

It must not be forgotten that the conclusions which we have drawn so far rest upon experimental evidence gained under specific conditions which have been described in detail above. Further work must show whether modifications of these conditions entail significant changes in the result.

Our knowledge concerning the various factors involved is limited. We do not know fully what influence the degree of sensitization plays; whether or not a phase of increased susceptibility to the action of x-rays precedes the establishment of a heightened resistance; how long this increased resistance persists; what the maximum resistance is which can be attained by this procedure, and other questions.

To some of these questions a partial answer can be given. As far as the degree of sensitization is concerned, one may state that the rabbits employed were highly sensitized. In earlier series of experiments, the same sensitizing procedure, dose, and period of incubation had been used by one of us, and in these animals the intravenous reinjection test had yielded a high mortality rate. It must always be remembered, however, that the degree of sensitization which a certain fixed method achieves, fluctuates more widely in rabbits than in guinea pigs. This may explain why we failed to produce any sign of protection in one rabbit of the sensitized group (Text-figs. 1 and 2).

As far as the maximum amount of x-rays is concerned, our results with 30 skin units indicate that this dose is fairly close to the limit of tolerance with the experimental procedure employed.

### *Specificity.*

The increased resistance to ordinarily lethal doses of x-rays which tissues may gain after a preliminary treatment with an undenatured foreign protein must be classed as a non-specific reaction, because the altered, abnormal response is called forth not by the sensitizing substance but by an utterly unrelated, physical agent. Such non-specific reactions after sensitization have been described and recognized for years. Heilner<sup>7</sup> in 1908 observed that serum-sensitized rabbits succumbed to an injection of 4 per cent sodium chloride which was practically harmless to normal controls. Davidsohn and

<sup>7</sup> Heilner, E., *Z. Biol.*, 1908, 1, 487.

Friedemann<sup>8</sup> showed that rabbits sensitized with bovine serum react with temperature elevations to doses of sodium chloride given subcutaneously or intravenously, which produce no such effect in normal rabbits. Richet<sup>9</sup> noted that dogs sensitized by actino-congestine or crépito-congestine vomited after smaller doses of apomorphine hydrochloride, injected intraperitoneally, than normal dogs.

Non-specific reactions have also been utilized therapeutically,<sup>10</sup> but in this respect great caution is advisable. It must be realized that the incorporation of an undenatured foreign protein entails consequences of whose manifestations we are largely ignorant, and therefore often no intelligent balance can be struck between the harm and benefit which the procedure affords the patient. This deficiency in our knowledge should theoretically be lessened by laboratory work on the lower animals. For these reasons the irrational use of vaccines and sera is to be discouraged. Such powerful drugs should be used only when nothing else suffices to gain the desired therapeutic end. A conscious distinction should be drawn between drugs whose single injection exerts a comparatively short effect and those whose single injection releases reactions which are often masked and persist for months and even years. Sera and vaccines therefore may not be employed with the same careless freedom, which, for example, characterizes the use of various synthetic compounds.

### *Mechanism of Protection.*

From the experimental data already presented and from the more obvious conclusions which we have so far drawn, no understanding of the underlying mechanism which brings about this increased resistance of the tissues to x-ray destruction can be reached. Some such

<sup>8</sup> Davidsohn, H., and Friedemann, U., *Berl. klin. Woch.*, 1909, xlv, 1120; *Arch. Hyg.*, 1909, lxxi, 42.

<sup>9</sup> Richet, C., *Compt. rend. Soc. biol.*, 1910, lxxviii, 820.

<sup>10</sup> For a review see Jobling, J. W., *The Harvey Lectures*, 1916-17, xii, 181; Miller, J. L., *J. Am. Med. Assn.*, 1921, lxxvi, 308; Cowie, D. M., *J. Am. Med. Assn.*, 1921, lxxvi, 310; Culver, H., *J. Am. Med. Assn.*, 1921, lxxvi, 311; Petersen, W. F., *J. Am. Med. Assn.*, 1921, lxxvi, 312.

basis can be obtained, however, if the results are considered in the light of a broad generalization of anaphylaxis. Such a generalization is the experimentally founded view<sup>11</sup> that an anaphylactic reaction is initiated when the anaphylactic antibody comes into contact with its antigen, during which process both antigen and antibody largely if not entirely disappear. If Text-figs. 1 and 2 are examined it will be observed that the only difference existing between the sensitized group and the sensitized-reinjected group is that the latter was subjected to an anaphylactic reaction 13 days after the x-ray treatment. In the sensitized-reinjected group, therefore, the anaphylactic antibodies had been removed more or less, while they were still present abundantly in the sensitized group. Since a majority of the sensitized group showed the marked resistance to massive doses of x-rays, while in the sensitized-reinjected group gangrene took place in the x-rayed area, it may be inferred that this protection is attributable to the anaphylactic antibodies which are present in the rabbits of the sensitized group, but which are not present, or at least not to the same functional degree, in the sensitized-reinjected group.

If the anaphylactic antibody is responsible for the protection to x-rays which the ears of the sensitized group exhibited, another inference may be drawn due to the fact that the x-ray treatment in the experiments was local. It follows that this protection must be assigned to the antibodies which are anchored to the tissue cells exposed to the x-rays and not to the circulating antibodies. This is shown clearly by the animals in the serum control group (Text-figs. 1 and 2). These animals had received one injection of horse serum, but this had been administered 13 days after the local x-raying of the ear. Within a short period an abundance of specific antibodies must have appeared in the circulation, and these necessarily must have traversed the capillary system of the x-rayed area of the ear. Yet practically no protection was conferred.<sup>12</sup> It appears, therefore, that the cells

<sup>11</sup> Anderson, J. F., and Frost, W. H., *J. Med. Research*, 1910, xxiii, 44; see also review by Wells, H. G., *Physiol. Rev.*, 1921, i, 51.

<sup>12</sup> A slight degree of protection is probably present. As shown in Text-fig. 1, the serum controls developed fenestration of the ears in 36 to 50 days while the normal controls attained the same state in a shorter time, 30 to 37 days. See other evidence in Text-fig. 2.

of an x-rayed area are unable to produce anaphylactic antibodies or to fix them, when present in the circulation, in sufficient amount to protect, provided that the x-ray treatment takes place before the injection of the antigen. The sensitized-reinjected group also supports this inference; in this group the antibodies formed as a result of the second injection of serum did not adequately replace those which were originally fixed in the x-rayed area but which were rendered inert by the anaphylactic reaction, though a certain measure of protection was observed (Text-figs. 1 and 2).

Finally, it may be inferred that the locally fixed anaphylactic antibodies (sensitized group) can be functionally removed by an anaphylactic reaction (sensitized-reinjected group) and the local protection which these fixed bodies gave against massive doses of x-rays is then largely abolished.<sup>13</sup>

It is probable that the increased resistance to x-rays conferred by a previous sensitization to the skin of rabbits will also be obtainable in man, and the procedure may thus be of utility in human therapeutics. Such a contingency will appear when malignant growths must be treated without the scalpel. Under these conditions the applicable dose of x-rays is directly limited by the resistance of the skin overlying the neoplasm for example, and a lethal dose for the cancerous tissue perhaps cannot be applied because it would also destroy the integument. This tentative proposal presupposes that the cancerous tissue does not acquire the same degree of resistance as the skin cells after sensitization, also that the heavy doses of x-rays do not ultimately produce malignant skin alterations. Concerning the first supposition, there is no knowledge available at present, but the experimental test is easily made; concerning the second, it may be said that no malignant changes in the skin of rabbits have been observed after a period of more than 300 days.<sup>14</sup> Finally, it may be stated that no objection can be urged against the parenteral employment of an undenatured foreign protein in such cases, because this effort is perhaps a last scientific attempt to help and it is therefore legitimate for the physician to invoke the aid of the protein molecule, fully conscious though he is that some or many of its various effects are not wholly desirable.

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<sup>13</sup> It is impossible to decide whether the moderate resistance of the sensitized-reinjected group is due to an imperfect removal of the anchored antibodies during the anaphylactic reaction or to anchorage of some antibodies subsequent to the anaphylactic reaction.

<sup>14</sup> This period of time in the rabbit is comparable to a much longer interval in the human subject, if we consider the relative length of life in the two species.



That sensitization with a foreign protein protects the skin from the harmful effects of a subsequent x-ray treatment is indicated by studies made by Hektoen. In a series of important observations<sup>15</sup> Hektoen studied the effect of massive doses of x-rays under various conditions upon the production of antibodies, the anaphylactic antibody not being included. His experimental material consisted of white rats, rabbits, and dogs, and the entire body of the animal was always subjected to the action of the x-rays. Hektoen established clearly that the time of x-raying with respect to the injection of antigen exerted a profound effect upon the antibodies. If the antigen was injected immediately after the preparatory x-ray treatment the production of antibodies was practically completely restrained. If, on the other hand, the x-raying was carried out at the height of antibody production (days or weeks after the antigen injection) no effect was noted on the antibody output.

The observations which interested us most, however, were as follows: When young puppies were x-rayed with strong doses of x-rays before they were injected with antigen (10 per cent rat or goat blood suspensions), severe burns of the skin were noted;<sup>16</sup> but if they were x-rayed about 7 days after the antigen injection, Hektoen apparently observed no burns, for he only states that now many dogs showed no disturbances of the general health.<sup>17</sup> If we are correct in this interpretation of Hektoen's work, our observations in this matter accord with his. We have not been able to find any other observations in the literature bearing upon this question.

### *Inflammation of the X-Rayed Area.*<sup>1</sup>

In the objective record of our results we have described three combinations in which inflammation of the x-rayed ear surface may appear. These three combinations, their distribution among the various experimental groups, and the duration of the process can be utilized to give further support to the antibody theory which has already been discussed.<sup>18</sup>

<sup>15</sup> Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 28. This article gives the references to Hektoen's earlier work. See also Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

<sup>16</sup> Hektoen, L., *J. Infect. Dis.*, 1918, xxii, 29.

<sup>17</sup> Hektoen,<sup>16</sup> p. 31.

<sup>18</sup> We have not included among the various types of inflammatory reaction the combination noted in Rabbit CX 18 (Table I). In this serum control rabbit the first inflammation exceptionally led at once to complete gangrene of a section of the x-rayed area. But 135 days later a subacute inflammation with crust formation developed and led to still another loss of tissue. This delayed second inflammation healed in 24 days (195 days after x-ray treatment). This type of reaction is probably allied to the delayed x-ray lesions which at times occur in the human subject months after the last treatment (Pfoerringer, S., Review in *Am. J. Roentgenol.*, 1917, iv, 642).

The three combinations of states are as follows:<sup>19</sup>

- (1) First inflammation.....gangrene.
- (2) First inflammation...healing.....second inflammation....gangrene.
- (3) First inflammation.....healing.

The distribution of these combinations is summarized in Table I. An examination of this table shows that the second type or combination (first inflammation—healing—second inflammation—gangrene) occurs only in the groups which had been subjected at one time or another to the injection of horse serum. It was never observed in normal rabbits treated with a destructive dose of x-rays.

The first combination (first inflammation—gangrene) occurred in all of the three normal controls; it also was observed in two additional normal control animals which had been x-rayed with 22 $\frac{2}{3}$  skin units. But in the serum animals this combination was only noted two times (Rabbits CX 18 and HSX 8).

The third combination (first inflammation—healing) was only observed in the sensitized group, in which the horse serum was administered previous to x-ray treatment. It occurred two times out of four experiments, in Rabbits HSX 12 and HSX 13. A third rabbit of this group (No. HSX 11) shows a very marked prolongation in the interval between recovery from the first inflammation and the onset of the second inflammation which led to gangrene.

From the occurrence of the second type of combination (inflammation—healing—inflammation—gangrene) in eight out of twelve rabbits which had been treated with horse serum (Table I), and from the failure of this combination to appear in five normal control animals to which no serum had been given, we may infer that the increased resistance of the x-rayed tissue evinced by the second combination of states is definitely ascribable to the serum treatment. In other words, the administration of serum at any time within the limits employed in the experiments changes the reaction of the x-rayed tissue from the first combination (inflammation—gangrene) to the second combination (first inflammation—healing—second inflammation—gangrene) in the majority of the treated rabbits.

<sup>19</sup> It should be remembered that the first inflammation was a mild acute form, while the second inflammation was subacute in character.

We conclude, therefore, that this change was due to a protective antibody action which was produced by the parenterally injected horse serum. From the data given in this section no inference can be drawn concerning the type of antibody which caused this change of reaction to the standard dose of x-rays. Such an inference, however, can readily be drawn if we use the occurrence of gangrene and the duration of the entire process (Text-figs. 1 and 2) as criteria, and in a preceding section evidence has been presented that the anchored, anaphylactic antibody may be considered the protective factor. It is therefore probable that the same anchored anaphylactic antibody is also responsible for the altered character of the local reaction which the serum-treated rabbits exhibit after x-ray treatment. What part, if any, is played by other types of antibodies in this matter cannot be determined by the data at hand.

On the basis of these considerations the various successions of conditions observed in the x-rayed areas of the rabbits may be explained as follows: The inflammation observed in normal control animals which ends in gangrene is the normal slow, destructive action of our standard x-ray dose (30 skin units). The tissues exposed show a mild, acute inflammation which leads to a complete destruction of a portion of the x-rayed area. How these tissue changes are produced by the physical agent, the x-rays, we do not know;<sup>20</sup> vascular changes such as we have described undoubtedly are involved in the process.

If rabbits are treated with horse serum parenterally and exposed to the same standard x-ray dose, the type of reaction changes, due to the presence of anaphylactic reaction bodies anchored in the x-rayed area, the latter factor depending upon the time or times when the serum is administered. If the serum is administered about 2 weeks after x-ray treatment or if it is injected before and after x-ray treatment in such a way that a general anaphylactic reaction results, the second combination of conditions (inflammation—healing—inflammation—gangrene) then appears in the x-rayed areas of a majority of the rabbits (Table I, serum control group, sensitized-reinjected group).

<sup>20</sup> For a good presentation of the various theories concerning the mode of x-ray action upon tissues, see Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 455.

The first inflammation now progresses to healing due to the presence of anaphylactic antibodies anchored in the x-rayed area. But this healing is only temporary, because the amount of locally available antibodies is too small or becomes functionally inert, and the slowly acting destructive forces gain the ascendancy over the reparative agencies. As a consequence the second inflammation appears which leads to a perforating gangrene. The second inflammation is subacute in character because the exposed area has been damaged by the x-rays, so that it can no longer react acutely to an inflammatory stimulus.

If, however, the serum is administered to a rabbit about 10 days previous to exposure to the standard dose of x-rays, the anaphylactic antibodies anchored in the x-rayed area may be sufficient in amount to protect that area for a long period or perhaps even indefinitely. The succession of conditions is then inflammation—healing—(Table I, sensitized group).

The explanation which we have given obviously only answers the question why the ordinary process of an x-ray action on tissues should be altered when the organism is treated with serum parenterally; how this alteration is produced we cannot say because it is not known how either the x-rays or the foreign protein exert their effects upon the tissue cells.

The results reported in this paper emphasize a precaution which ought to be observed in all animal experimentation. Since mere sensitization with an alien protein alters the reactivity of an organism not only towards the specific alien protein itself, but also towards an unknown number of other, unrelated substances or even physical agents, it is obvious that sensitized animals cannot serve as normal controls until it has been demonstrated that both the sensitized and normal animals react to the same agent in the same manner and to the same degree. Discarded animals which have been subjected experimentally to the action of undenatured proteins of bacterial, protozoan, metazoan, or vegetable origin should be used in identified groups when they are reemployed for an investigation. Failure to respect this precaution perhaps explains some of the discordant results obtained in diverse studies of the same problem. It is further possible that some of the erratic fluctuations in the degree of a reaction observed in a group of supposedly normal animals have their cause in an unsuspected proteinization of the abnormal reactors. The possibility or even probability of unwittingly employing proteinized mammalian material cannot be denied, for most investigators are compelled to rely upon dealers for their animal stock. In com-

pensation for this uncertainty, we may perhaps look upon the abnormal reactors among a group of animals as indicators of a possible proteinized state, and thus gain a working hypothesis which may add to our knowledge of non-specific phenomena.

#### SUMMARY.

When a fixed area of the ears of rabbits is subjected to the action of a standard destructive dose of x-rays (30 skin units) the type of reaction resulting depends upon the previous treatment of the rabbit. (1) In normal rabbits a mild acute inflammation develops in the x-rayed area which leads at once to a perforating gangrene within an average of 15 days. (2) If rabbits are x-rayed and about 2 weeks later injected with horse serum for the first time, a mild acute inflammation appears which heals for a time; then a second, subacute inflammation sets in which leads to a perforating gangrene. The average time of the process from the first inflammation to gangrene is 32 days. (3) If rabbits are sensitized with horse serum and 10 days later are exposed locally to the standard dose of x-rays, the ensuing ear reaction is either similar to the second reaction described above, except that it may last up to 110 days, or the first inflammation leads to a healing which may be apparently permanent (340 + days). (4) If rabbits are first sensitized with horse serum, exposed locally to the standard dose of x-rays 10 days later, and 13 days after the x-ray treatment reinjected with horse serum, the reaction of the x-rayed area of the ears is in general similar to the second reaction described above (inflammation—healing—inflammation—gangrene). The average time of the whole process is about 42 days.

On the basis of the general hypothesis that an anaphylactic reaction is initiated in the body when the specific antibody meets its antigen, and that both antibody and antigen are rendered more or less functionally inert by their interaction, the following inferences may be drawn from our experimental results. (1) The protection from the effects of a standard destructive dose of x-rays which a previous sensitization confers, is referable to the presence of anaphylactic antibodies in the x-rayed area. (2) This protection is largely due to the anaphylactic antibodies which are anchored in the x-rayed area, and not to those which are free in the circulation. (3) An anaphylactic reaction

renders the anchored anaphylactic antibodies largely impotent as protective factors against the standard destructive x-ray dose, even though sensitization preceded exposure to the x-rays. (4) An area treated with the standard destructive dose of x-rays is unable to produce or to anchor a sufficient amount of anaphylactic antibodies for protection from necrosis, when the x-ray treatment precedes the sensitization, or when the locally anchored anaphylactic antibodies are rendered functionally inactive by a general anaphylactic reaction.

It is possible that the procedure of increasing the resistance of the skin to a destructive dose of x-rays by means of a previous sensitization with protein may be applicable in the treatment of certain types of inoperable disease, when it is important to use massive doses of x-rays.

Animals which have been sensitized, or sensitized and reinjected with any undenatured alien protein, should not be reemployed as normal controls in any investigation unless trial has shown that these proteinized animals react quantitatively and qualitatively like normal animals.

The presence of an abnormal reactor in a group of supposedly normal animals may be an indication of a previous proteinization.

#### EXPLANATION OF PLATES.

The photographs of the rabbit ears were taken by transillumination while the blood vessels were in a dilated state. The time of photographing was 181 days after x-raying. The vessel traversing the middle of the ear is the central artery and in most figures its bifurcation near the upper pole of the ear can be seen. The marginal ear vein is also usually clearly visible. The x-rayed area of the right ear is shown as a bald quadrilateral space. Unfortunately the normal control rabbits were not photographed.

#### PLATE 100.

FIG. 1. Serum control group; Rabbit CX 16. Perforating gangrene occurred about 50 days after x-ray treatment.

FIG. 2. Serum control group; Rabbit CX 17. Perforating gangrene occurred about 50 days after x-ray treatment, but the process exceptionally is not yet complete although 181 days had passed since the x-raying. This is shown by the slowly healing, superficial ulcer to the right of the perforation, appearing as a black patch in the photograph.

## PLATE 101.

FIG. 3. Sensitized group; Rabbit HSX 12. This figure shows that the bald, x-rayed surface is perfectly smooth with no perforation or crusts. The central artery in the x-rayed area is markedly narrowed. This area has remained in the same state for over 340 days after the date of x-ray treatment.

FIG. 4. Sensitized group; Rabbit HSX 13. The x-rayed area is intact and bald and the central artery shows clearly a partial stenosis. The small black spot represents a slight hemorrhage from a collection of fine, tortuous, superficial vessels at that point. The x-rayed area has remained in this condition now for more than 340 days.

## PLATE 102.

FIG. 5. Sensitized and reinjected group; Rabbit HSX 5. The perforating gangrene took place 85 days after x-ray treatment. This figure shows clearly how both the central artery and the marginal ear vein are narrowed in the x-rayed field.

FIG. 6. Sensitized and reinjected group; Rabbit HSX 7. Perforating gangrene took place 62 days after x-ray treatment. The partial stenosis of a vein in the x-rayed area is shown to the right of the perforation.

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FIG. 1.

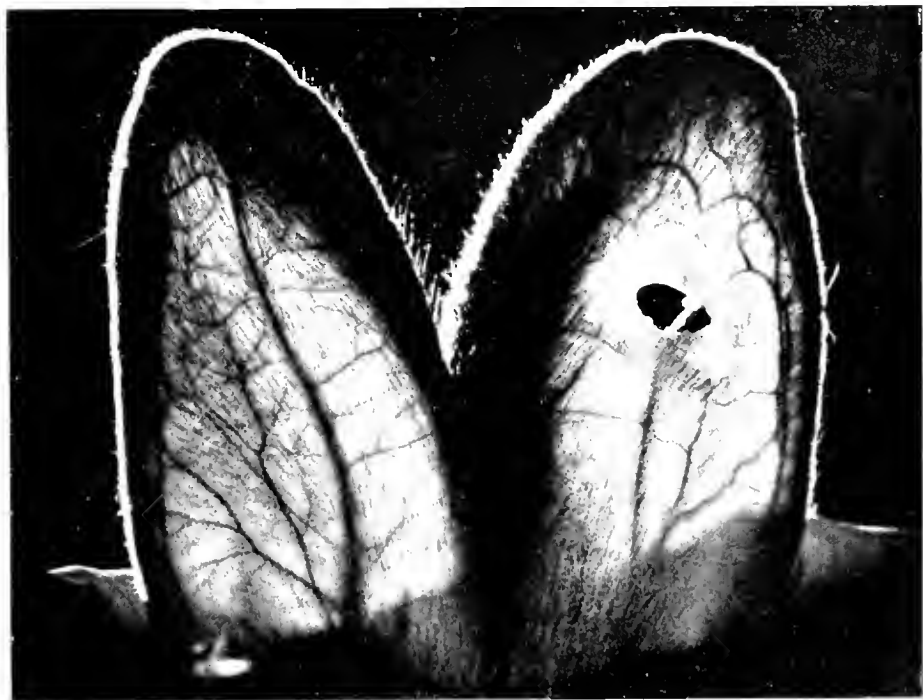


FIG. 2.

Am. and Withcher, Roentgenograms of the lungs.



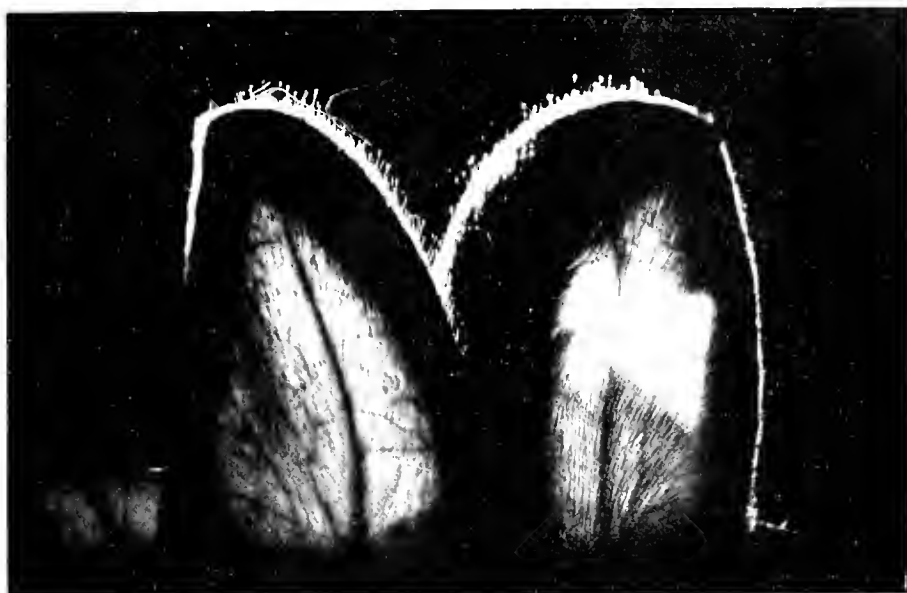


FIG. 3.



FIG. 4.

(Auer and Witherbee: Reaction of normal skin to x rays)



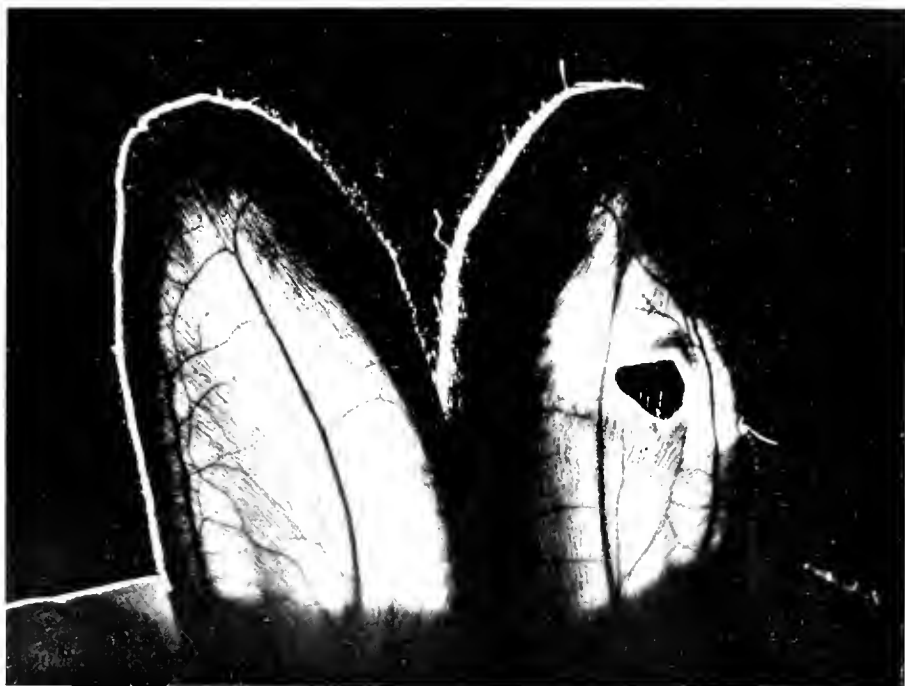


FIG. 5.

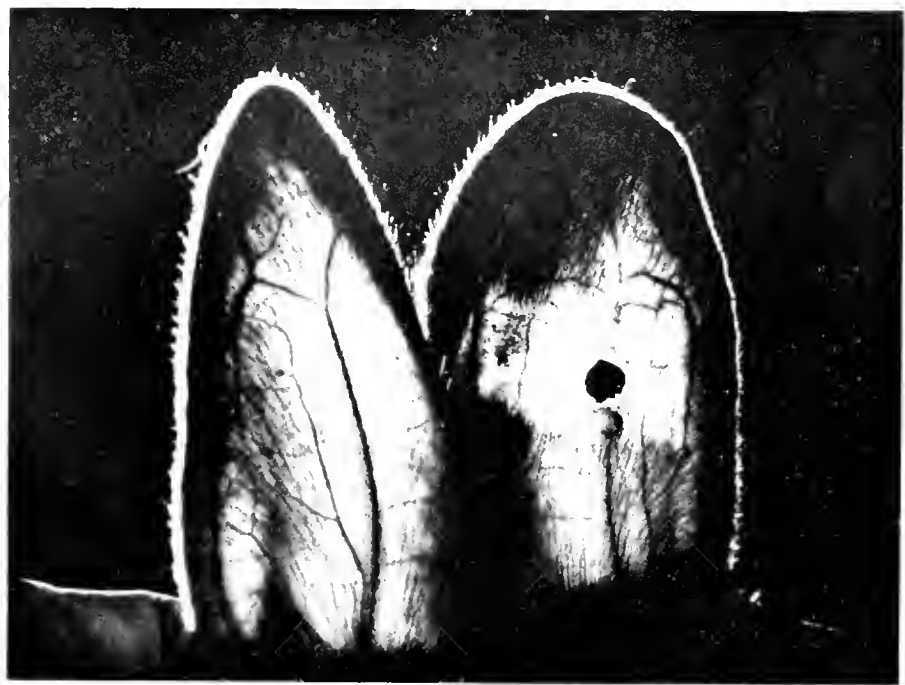


FIG. 6.

(Ames and Witherbee, Reaction of normal skin to x-ray.)



# EFFECT OF SMALL DOSES OF X-RAYS ON HYPERTROPHIED TONSILS AND OTHER LYMPHOID STRUCTURES OF THE NASOPHARYNX.\*

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PLATES 103 TO 108.

(Received for publication, March 11, 1921.)

In work carried on by us over several years we have extended the original observation of Heinicke<sup>1</sup> on the susceptibility of lymphoid tissue to x-rays and have shown other ways in which the x-rays may be employed as a therapeutic agent. It may be regarded now as established that the lymphoid tissue is more highly susceptible to x-rays than any of the structures of the body, except the sex glands, and that by suitable exposures it is possible to remove almost all of the lymphoid tissue without inducing detectable changes in other organs or tissues.<sup>2</sup> When the doses of x-rays are properly graded even the total number of polymorphonuclear leucocytes remains unaffected while the lymphocytes fall to a point at which few are seen in the circulating blood.<sup>3</sup>

The use of x-rays for reducing enlarged lymphoid organs is not new, but in the past the doses employed for the purpose have been large and thus have approached the danger point so closely that it has

\* A preliminary report was made of this work before the American Society for Clinical Investigation and published in the Proceedings of the Society (Murphy, Jas. B., *J. Am. Med. Assn.*, 1920, lxxiv, 1738).

<sup>1</sup> Heinicke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

<sup>2</sup> Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459. Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

<sup>3</sup> Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

been resorted to only in extreme instances. If, as our work indicates, extensive reduction of lymphoid tissue can be induced by small doses of x-rays, well within the safety limit, there is no reason why x-rays should not be used as a therapeutic agent in a variety of conditions. For obvious reasons the tonsil has been selected for the purpose of testing this point.

Histological examination of the tonsil shows it to have a structure similar to that of other lymphoid glands, with the exception of the fact that it is covered on one side by mucous membrane with crypts dipping down from the surface. These crypts have been described as natural test-tubes for the growth of bacteria. Whether or not the presence of pathogenic organisms in the crypts is the source of hypertrophy of the organ or whether the hypertrophy arises from another set of conditions is a moot point. It is, however, agreed that enlarged tonsils with resultant poorly drained crypts have a pathological significance.

In addition to the enlargement of the tonsil, other lymphoid deposits showing hypertrophy occur through the mucous membrane of the pillars of the fauces and as masses back of the posterior pillars. These structures also become pathologically altered in much the same way as the tonsil. They are not subject to surgical removal as is the tonsil but since they are made up of lymphoid cells are subject to influence by x-rays.

The following study was undertaken in order to test the effect of small doses of x-rays on the tonsil and other lymphoid deposits of the nasopharynx.

### *Technique.*

The individuals to be treated are placed on a table with the head tilted so that the axis of the x-rays may pass under the angle of the jaw into the region of the tonsil. The area exposed on each side of the neck is about 3 inches square, the surrounding surface being covered with heavy sheet lead. The factors governing the dose of x-rays to each area were as follows: 8 inch spark-gap measured between points, 5 milliamperes, 10 inches distance from the target to the highest point of skin exposed; the time varies from 3 to 7 minutes, depending on the age of the individual, and the x-rays were filtered through 3 mm. of aluminum. The approximate value of this dose



is from one to one and three-quarters skin units. After an interval of a few weeks this treatment may if necessary be repeated with safety.<sup>4</sup> To insure immobility in young children a special board has been used with retaining straps and the child's head secured by means of a gauze bandage.

When excessive adenoid tissue was present a third area was exposed; namely, the back of the neck, just below the posterior occipital region with the head tilted forward. But this site of entry for the x-rays is less favorable, as will be indicated later in this paper.

### *Material.*

This report is based on the study of 46 individuals ranging in age from  $3\frac{1}{2}$  to 45 years and observed 1 month or longer after treatment. About 40 other individuals were treated, but as they did not return for examination they have not been included. The condition of the tonsils was noted in each individual and a drawing showing the size made by an artist independent of the examining physician. The state of the tonsils on first examination varied from that of simple hypertrophy to the enlarged organ with ragged surface and deep crypts containing exudate, or the small pathologically altered tonsil associated with symptoms of systemic disease. No individuals were treated at a time when the throat showed signs of acute inflammation.

The histories of eleven selected typical cases will be given and drawings of some of these, showing the progressive changes in the throat.

*Case A.*—J. L. L., white, male; age 19 years (Fig. 1).

Mar. 15, 1920. Throat: The tonsils, small and buried, the left larger than the right, ragged in appearance; numerous crypts containing exudate. Lymphoid

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<sup>4</sup> In a later study Witherbee has recommended the use of fractional doses, four or more if necessary, treatments being given at 2 week intervals (Witherbee, W. D., *Am. J. Roentgenol.*, 1921, viii, 25). The factors of this dose are, 7 inch spark-gap measured between points, 5 milliamperes, 10 inches distance, time of exposure 3 minutes and 18 seconds; filtered through 3 mm. of aluminum. The principal advantage of this method is that it makes the treatment more flexible and the individual may be given more nearly the amount of x-rays necessary to induce the desired result.

tissue behind pillars increased in amount; large amount of adenoid tissue. Enlarged cervical lymph nodes. Bacteriological examination:<sup>5</sup> Right tonsil 50, left tonsil 150 colonies of hemolytic streptococci; vault none. X-rays: Three areas, right and left sides of neck and posterior occipital region, exposed to filtered x-rays; spark-gap 8 inches, milliamperes 5, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 22. Tonsil tissue markedly reduced and glazed and pale in appearance; lymphoid tissue behind pillars pale and smooth. Bacteriological examination: Right tonsil 50, left 150, and vault 50 colonies of hemolytic streptococci.

Apr. 5. Tonsil pale; edges of crypts inverted; no exudate can be pressed from tonsil. Adenoids considerably reduced; smooth and pale. Bacteriological examination: Right tonsil 100, left tonsil 50, and vault 150 colonies of hemolytic streptococci.

Apr. 26. Little visible tonsil tissue, of smooth appearance; no exudate; adenoid tissue small in amount, smooth and regular. Bacteriological examination: No hemolytic streptococci found.

*Case B.*—E. S., white, male; age 26 years (Fig. 2).

Dec. 10, 1919. Throat: Medium sized tonsils with deep crypts full of exudate; ragged inflamed surface. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 4, 1920. Tonsil shows marked shrinkage; some exudate present. X-rays: Two areas, right and left side of neck, exposed to x-rays in same dosage as above.

Apr. 4. Tonsils small; smooth surface; no exudate.

Mar. 5, 1921. Tonsils small with smooth surface; no exudate.

*Case C.*—H. W., white, male; age 14 years.

Mar. 17, 1920. Throat: Tonsils very large, buried; irregular surface; numerous crypts with yellowish exudate. Mass of lymphoid tissue back of pillars. Adenoid tissue, large mass covering surface of vault and fossa. Bacteriological examination: Right tonsil no colonies, left 50, and vault 50 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 24. Tonsils show decided reduction in size; pale, smooth surface; on pressure some whitish secretion. Adenoid tissue considerably reduced and of smooth, clean appearance. Bacteriological examination: Tonsils no colonies, vault 50 colonies of hemolytic streptococci.

Mar. 31. Tonsils show further reduction in size; smooth and pale; no exudate on pressure. Adenoid tissue pale, smooth, and clean; more normal in appearance. Bacteriological examination: Tonsils and vault show no hemolytic organisms.

Apr. 7. Improvement continues. Lymphoid tissue along posterior pillars has entirely disappeared.

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<sup>5</sup> It is obvious that the number of colonies is roughly indicative only, as the quantity of material inoculated and part of organ touched with the loop are not subject to accurate control.

June 14. Tonsils flat; pale smooth surface; adjacent mucous membrane pale; edges of crypts inverted and crypts show retraction. Adenoid mass materially reduced. Bacteriological examination: No cultures taken.

*Case D.*—S. A., white, male; age 31 years.

Apr. 7, 1920. Throat: Tonsils moderately enlarged, buried; numerous crypts; purulent fluid on pressure. Marked hypertrophy of lymphoid tissue along posterior pillars. No adenoid tissue. Bacteriological examination: Right tonsil 200, left tonsil 200, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays. Spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 12. Tonsils show some reduction; pale and smooth; very little exudate on pressure. Marked reduction in lymphoid tissue on posterior pillars. Bacteriological examination: Right tonsil 150, left tonsil 200, and vault no colonies of hemolytic streptococci.

Apr. 19. Tonsils markedly reduced, smooth, pale, normal in appearance; less exudate on deep pressure. Further reduction in amount of lymphoid tissue on pillars. Bacteriological examination: Right tonsil 50 colonies, left tonsil no colonies, and vault 50 colonies of hemolytic streptococci.

May 10. Tonsils further reduced; mucous membrane of tonsil and pillars smooth and pale; edges of crypts rounded; small amount of exudate on deep pressure. Lymphoid deposit on pillars has practically disappeared. Bacteriological examination: No hemolytic streptococci found.

*Case E.*—J. V. K., white, male; age 15 years (Fig. 3).

Mar. 31, 1920. Throat: Tonsils large with ragged, irregular surface; numerous crypts with exudate. Large irregular mass of adenoid tissue. Bacteriological examination: Right tonsil 150 colonies of hemolytic staphylococci, 50 colonies of hemolytic streptococci; left tonsil 50 colonies of hemolytic streptococci; vault 100 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Apr. 7. Tonsils slightly reduced; less ragged in appearance. Adenoids show some reduction. Vault contains mucopurulent discharge. Bacteriological examination: Right tonsil 100, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils markedly reduced; surface irregular, ragged, and congested. Adenoid tissue reduced; smoother surface. Bacteriological examination: No hemolytic organisms found.

Apr. 21. Tonsils show still further reduction; surface smooth; edges of crypts inverted. Adenoid tissue markedly reduced; smooth pale surface. Bacteriological examination: No hemolytic organisms found.

Apr. 26. Tonsils flat; pale, smooth, clean surface; edges of crypts round and inverted. Bacteriological examination: No hemolytic organisms found.

May 24. Tonsils normal in appearance. Adenoid mass reduced in size.

Sept. 29. Tonsils very small; smooth, pale surface. Adenoids greatly reduced and normal in appearance.

*Case F.*—S. V. M., white, male; age 10 years.

Mar. 24, 1920. Throat: Tonsils large, partly buried; irregular, ragged surface; numerous crypts filled with thick yellow exudate. Lymphoid tissue behind pillars markedly hypertrophied. Large irregular mass of adenoid tissue covered with purulent exudate. Bacteriological examination: Right tonsil 200, left tonsil 100, and vault no colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils reduced in size; edges of crypts inverted; less exudate. Pharynx had dull red glazed appearance. Adenoid tissue considerably reduced; pale; less exudate. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Apr. 7. Tonsils markedly reduced; some surface secretion; crypts much cleaner. Adenoid tissue still further reduced. Bacteriological examination: No hemolytic organisms found.

Apr. 14. Tonsils very markedly reduced; surface smooth and clean; edges of crypts inverted and smooth; no exudate on deep pressure. Adenoid tissue smooth; normal appearance. Lymphoid tissue back of posterior pillars practically disappeared. Bacteriological examination: No hemolytic organisms found.

Sept. 29. Tonsils show further reduction; normal in appearance; edges of crypts smooth and inverted. Adenoid tissue still present; small amount of exudate on pressure.

*Case G.*—J. W., white, male; age 17 years (Fig. 4).

Mar. 24, 1920. Throat: Tonsils very large; ragged and congested. Considerable hypertrophy of lymphoid tissue behind posterior pillars. Bacteriological examination: Right tonsil 200, left tonsil 150, and vault 200 colonies of hemolytic streptococci. X-rays: Three areas, right and left side of neck and posterior occipital region, x-rayed; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 31. Tonsils showed marked reduction; pale and smooth. Bacteriological examination: Right tonsil 100, left tonsil 50, vault 100 colonies of hemolytic streptococci.

Apr. 14. Tonsils show still further reduction; smooth, normal appearance. Lymphoid tissue behind posterior pillars entirely gone. Bacteriological examination: No hemolytic organisms found.

June 28. Tonsils small, smooth, pale; edges of crypts inverted and translucent.

Sept. 13. Tonsils show some further reduction; white bands around edges of inverted crypts; right tonsil shows some cheesy deposits, easily removed on pressure. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Feb. 17, 1921. Tonsils small, flat; surface smooth and pale; no exudate. Bacteriological examination: No hemolytic organisms found.

*Case H.*—J. Z., white, male; age 21 years (Fig. 5).

Mar. 10, 1920. Throat: Tonsils moderately enlarged; ragged surface; numerous crypts. Left tonsil has large fossa filled with purulent exudate. Moderate sized mass of adenoid tissue, irregular, covered with whitish secretion. Bacteriological examination: Right tonsil no hemolytic organisms; left tonsil 50 colonies of hemolytic streptococci and 200 colonies of hemolytic staphylococci; vault 50 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci. X-rays: Three areas, right and left side of neck and posterior occipital region, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 17. Tonsils distinctly reduced; cleaner in appearance. Adenoids cleaner. Bacteriological examination: No hemolytic organisms found.

Mar. 31. Tonsils reduced; still some exudate. Adenoid tissue reduced, pale; less exudate.

Apr. 28. Tonsils show marked reduction; smooth, pale surface; crypts clean; no exudate on deep pressure.

June 14. Tonsils show still further reduction; no exudate from right tonsil, small amount from left on deep pressure. Adenoids possibly reduced.

Sept. 13. Tonsils small, smooth, pale; on deep pressure still some exudate; edges of crypts markedly inverted. Adenoid tissue still present.

Mar. 11, 1921. Tonsils small; normal in appearance.

*Case I.*—V. S., white, male; age 14 years (Fig. 6).

Mar. 3, 1920. Throat: Tonsils large, ragged; numerous crypts full of pus. Large mass of lymphoid tissue behind posterior pillars. Large mass of adenoid tissue. Bacteriological examination: Right tonsil 100 colonies of hemolytic streptococci and 50 colonies of hemolytic staphylococci; left tonsil 50 each of hemolytic streptococci and staphylococci; vault 50 colonies of hemolytic streptococci and 150 of hemolytic staphylococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 10. Tonsils reduced in size; smooth surface; less exudate on pressure. Lymphoid masses behind posterior pillars markedly reduced; smooth glazed appearance. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms found.

Mar. 17. Tonsils still further reduced; pale, clean. Bacteriological examination: No hemolytic organisms found.

Mar. 24. Tonsils smaller; smooth surface. Further reduction in lymphoid tissue back of pillars. Bacteriological examination: No hemolytic organisms found.

Apr. 28. Tonsils show marked reduction in size; pale and smooth; no injection of mucous surfaces; no exudate on deep pressure; edges of crypts smooth. Lymphoid tissue behind posterior pillars practically disappeared. Adenoid mass reduced. Bacteriological examination: No hemolytic organisms found.

Sept. 13. Tonsils small and normal in appearance; no exudate on deep pressure; edges of crypts smooth and inverted; mucous surfaces show no injection. Lymphoid deposits back of pillars practically gone. Adenoids reduced but still large.

*Case J.*—E. C., white, male; age 16 years.

Mar. 22, 1920. Throat: Tonsils large; numerous crypts filled with yellowish exudate; large amount of cheesy material on pressure. Hypertrophy of lymphoid tissue on posterior pillars. Large irregular mass of adenoid tissue covered with yellowish exudate. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 6 minutes, filtered through 3 mm. of aluminum.

Mar. 29. Tonsils considerably reduced; smooth and pale; no exudate on pressure. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 200, left 150, and vault 150 colonies of hemolytic streptococci.

Apr. 5. Tonsils further reduced; pale and smooth; edges of crypts smooth; slight exudate on pressure. Adenoids markedly reduced; pale, smooth, and clean. Bacteriological examination: Right tonsil 50, left 50, and vault no colonies of hemolytic streptococci.

Apr. 14. Tonsils and adenoids markedly reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

May 10. Tonsils small; normal in appearance; mucous surfaces pale; edges of crypts rounded and smooth; no exudate on deep pressure. Lymphoid tissue on posterior pillars has practically disappeared. Adenoid mass considerably reduced in size.

June 14. Tonsils normal in appearance. Adenoids very small, pale, smooth mass.

Sept. 29. Tonsils and adenoids small and normal in appearance; surface smooth; no exudate.

*Case K.*—J. F., white, male; age 16 years.

Mar. 8, 1920. Throat: Tonsils large, ragged; numerous crypts. Large mass of adenoid tissue. Enlargement of cervical glands. Bacteriological examination: Right tonsil 100, and left tonsil 50 colonies of hemolytic streptococci. X-rays: Two areas, right and left side of neck, exposed to x-rays; spark-gap 8 inches, milliamperes 5, distance 10 inches, time 7 minutes, filtered through 3 mm. of aluminum.

Mar. 15. Tonsils reduced; smooth, glazed surface. Adenoids considerably reduced; smooth and glazed. Bacteriological examination: Right tonsil 100, left tonsil 150, and vault no colonies of hemolytic streptococci.

Mar. 29. Tonsils further reduced; pale and smooth. Adenoids also reduced; pale and smooth. Bacteriological examination: No hemolytic organisms found.

Apr. 5. Right tonsil more reduced than left; surface pale and smooth. Adenoid mass pale, clean.

Apr. 12. Both tonsils further reduced; crypts inverted. Adenoid tissue considerably reduced. Bacteriological examination: Right tonsil 50 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

May 10. Tonsils markedly reduced; surface normal; edges of crypts rounded and inverted; on deep pressure still slight amount of exudate. Lymphoid tissue behind posterior pillars completely disappeared. Adenoid tissue reduced but still large. Enlarged cervical glands somewhat smaller. Bacteriological examination: Right tonsil 5 colonies of hemolytic streptococci, left tonsil and vault no hemolytic organisms.

Sept. 29. Tonsils markedly reduced; normal in appearance; edges of crypts inverted, pale; no exudate. Adenoid tissue still present.

Table I shows the class of cases which have been treated with x-rays and the result of this treatment.

In general it may be said that the reduction in size of the tonsils and other lymphoid deposits in and behind the pillars of the fauces becomes clearly evident about 2 weeks after treatment and increases for 1 to 2 months. As the tonsil shrinks the crypts open and drain, and finally the edges become inverted and the surface grows smooth and healthy in appearance. The small lymphoid deposits in the pillars and the larger accumulation frequently present behind the posterior pillars of the fauces disappear promptly after the treatment and at the same time the injection of the vessels subsides.

The adenoid tissue is, as expected in view of the portal of entry used for the x-rays, not so uniformly reduced as the tonsils. This particular aspect of the problem of reducing excessive lymphoid tissue in the nasopharynx through x-rays is one to which in the near future especial attention will need to be given.

#### *Bacteriological Examination.*

Cultures were taken from the throats of 40 of the 46 individuals before and at intervals after treatment. The material for culturing was obtained from the crypts of the right and left tonsils, by means of a platinum wire about 3 inches long at the end of which was a small loop 2 mm. in diameter, bent at a right angle to the main piece of wire. The tongue was pressed down firmly with a tongue depressor, and the wire introduced into the crypt of the tonsil with a "stab and twist" motion. Caution was used to avoid contamination from

TABLE I.

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment. <i>mos.</i>	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
1	31	M.	++++*	Numerous crypts; mucopurulent exudate; considerable lymphoid tissue behind pillars.	2	++	Mucous membrane smooth; lymphoid tissue behind pillars almost gone; general appearance normal.	None.	
2	35	M.	+++	Soft, friable; numerous crypts; thin purulent exudate.	1	+	Edge of crypts smooth; normal.	Small.	Some reduction.
3	13	M.	+++++	Ragged, inflamed; exudate.	6	++	Smooth, pale; right tonsil still not normal.	Large.	Little affected by treatment.
4	29	M.	+++++	Ragged; deep crypts.	4	++	Crypts smooth and shallow; normal appearance.	Large.	Considerable reduction.
5	25	F.	+++++	Not markedly abnormal.	3	++	Edges of crypt inverted; normal.	Small.	Reduction.
6	16	M.	+++++	Numerous crypts; yellow, cheesy exudate; large mass of lymphoid tissue posterior to pillars.	6	++	Surface normal; no exudate in crypts; lymphoid masses posterior to pillars gone.	Large; covered with exudate.	Marked reduction; normal surface.



7	7	M.	+++++	Ragged; crypts contain mucopurulent exudate.	5	+++	Improvement; tonsils still not normal.		
8	15	F.	+++++	Numerous crypts with purulent exudate.	1½ <i>wks.</i>	+++	Normal appearance; crypts inverted.	Large.	Reduction.
9	30	F.	+++	Ragged, inflamed; numerous crypts; exudate.	3 <i>mos.</i>	++	Clear, normal.	Medium.	Reduction.
10	?	M.	++++	Deep crypts with cheesy exudate; mass behind pillars.	5	++	Some exudate on deep pressure; surface normal; posterior pillar deposits absent.	Medium.	Reduction.
11	7	F.	++++	Ragged; deposits posterior to pillars.	5	++	Smooth; normal appearance; deposits posterior to pillars gone.	Large.	Marked reduction.
12	6½	F.	+++++	Numerous crypts with ragged edges.	5	+	Normal.	Small.	No reduction.
13	15	M.	+++++	Ragged, soft, congested.	1	++	Absolutely normal appearance.	Medium.	Marked reduction.
14	16	M.	+++++	Numerous crypts; ragged edges; deposit behind pillars.	6½	++	Normal appearance; deposits behind pillars gone.	Large.	No marked reduction.
15	3	M.	+++++	Ragged.	1	++	Much improved.	Very large.	Reduction.
16	6	F.	+++++	Congested; numerous crypts; ragged; deposits posterior to pillars very large.	5½	++	Absolutely normal; deposit back of pillars gone.	Medium.	No marked reduction.

\* + + + + + very much enlarged; + + + + much enlarged; + + + medium enlargement; + + approximately normal; + small; - not visible.

TABLE I—Continued.

			Tonsils.					Adenoids.	
Individual No.	Age.	Sex	Size before treatment.	Condition.	Length of time under observation after treatment.	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
17	5 yrs.	M.	+++++	Ragged.	mos. 1½	+++	Edges of crypts inverted; smooth surface.	Very large.	No marked reduction.
18	49	M.	+++++	Ragged; large deposit back of pillars.	6	++	Normal; posterior pillar deposits greatly reduced.	Large; ragged.	Some reduction; normal appearance.
19	28	F.	+++++	Yellow, cheesy exudate; numerous crypts.	2½	+	Normal.	Medium.	Definite reduction.
20	28	F.	++	Ragged; numerous large crypts.	1	+	Normal; surface smoothed out.	Medium; mucopurulent exudate.	Reduction; no exudate.
21	15	M.	+++++	Ragged; large crypts.	6	+	Normal; inversion of crypt.	Large; irregular.	Reduction; smooth.
22	35	M.	++++	Inflamed; ragged; numerous crypts; exudate.	1	++	Normal appearance; smooth, clean surface.		
23	7	F.	++++	Ragged.	2	+	Normal appearance.	Large, irregular.	Some reduction.
24	6	F.	+++++	Ragged; numerous crypts; mucopurulent exudate.	2	++	Smooth, normal surface.		
25	26	M.	++++	Cheesy exudate.	3	+	Perfectly normal.	Small.	Reduction.

	26	19	M.	+	Ragged; numerous crypts.	1	—	Normal appearance; crypts free from exudate.	Large.	Great reduction.
27	10	M.	++++	Ragged; purulent exudate in crypts.	6	+	+	Normal clean surface; crypts inverted.	Large.	Slight reduction.
28	7	M.	+++	Numerous crypts; pale and ragged.	2	++	++	Normal; crypts inverted.	Large.	No marked reduction.
29	33	M.	+++	Purulent exudate; crypts irregular with cheesy deposit.	10	+	+	Normal.	Medium.	Reduction.
30	6	F.	++++	Irregular ragged surface; mucous membrane injected.	2	++	++	Clean; mucous membrane pale.	Large.	No marked reduction.
31	4	F.	+++	Ragged, congested.	2	++	++	Surface clear.†	None.	Reduction.
32	17	M.	++++	Irregular surface, congested; thick yellow exudate; deposits posterior to pillars.	6	++	++	Surface pale and clean; normal.	Small.	
33	35	M.	+++	Yellow exudate in crypts; lymphoid tissue back of pillars.	1½	+	+	Normal appearance.		
34	45	F.	+++	Cheesy exudate.	5	+	+	Surface clear; one tonsil still has some exudate on deep pressure.		
35	31	M.	++++	Ragged, congested; purulent exudate and cheesy deposits.	1	++	++	Normal appearance.	None.	
36	14	M.	++++	Crypts contain pus; lymphoid deposits back of pillars.	6	+	+	Normal appearance; deposits back of pillars reduced.	Large.	Reduction.

† Later had an attack of tonsillitis.

TABLE I—*Concluded.*

Individual No.	Age.	Sex.	Tonsils.					Adenoids.	
			Size before treatment.	Condition.	Length of time under observation after treatment <i>mos.</i>	Size at last examination.	Condition after treatment.	Size and condition before treatment.	Size and condition after treatment.
37	36 yrs.	F.	++	Mucopurulent exudate; lymphoid tissue back of pillars.	5	+	Normal.	Small.	No marked reduction.
38	17	M.	++++	Ragged, congested; lymphoid deposit back of pillars.	8	+	Smooth, pale; some exudate on deep pressure; lymphoid deposits gone.	None.	
39	21	F.	+++	Ragged, congested.	3	+	Normal.	None.	Little reduction.
40	30	F.	++++	Not abnormal.	1	+	Normal.	Small.	
41	23	F.	++	Mucopurulent exudate; cheesy deposits.	5	+	Smooth surface, normal appearance.	None.	
42	14	F.	++	Mucopurulent exudate; cheesy deposits; soft and friable.	5	+	Surface pale and normal; some exudate on deep pressure.	Large.	Little reduction.
43	14	M.	+++	Yellowish exudate.	3	+	Normal.	Large.	Reduction.
44	21	M.	++	Cheesy exudate.	1	+	Normal appearance; still some exudate on deep pressure.	Small.	Little reduction.
45	21	M.	++++	Subacute inflammatory condition.	8	+	Normal appearance; small amount of exudate on deep pressure.	Small.	Little reduction.
46	25	M.	+++	Inflamed; covered with mucopurulent exudate.	14	+	Absolutely normal.	None.	

the tonsil surfaces and the saliva. The material, so obtained, was placed on a blood agar plate, and gently spread over the surface with another platinum wire. The plates were then placed in the incubator at 37.5°C. Examinations of the plates and records of the findings were made after 24 and 48 hours of incubation.

Cultures were also taken from the nasopharyngeal vault by means of a very thin, semicircular, platinum wire, at the end of which was a small loop similar to that of the wire used in culturing the crypts of the tonsils. After firmly depressing the tongue, this wire was introduced into the vault directly back of the nose. The examination of the blood agar plates of these cultures was also made after incubating for 24 and 48 hours.

No attempt was made to differentiate the more common organisms usually found in the throat, such as pneumococcus, *Streptococcus viridans*, *Staphylococcus albus*, and *Staphylococcus aureus*. In distinguishing between hemolytic streptococcus and hemolytic staphylococcus, subcultures and stained films were resorted to. When the colony ranged in size from a pin-point to a small pin-head, and the area of hemolysis around the colony was from three to five times greater, streptococcus was usually found. Colonies which were as large as pin-heads or greater in diameter and about which the zone of hemolysis was very slight, appearing as a small halo, were usually staphylococcus.

36 of the 40 individuals showed hemolytic organisms to be present. Of these, seven became negative 1 week after treatment, fourteen after 2 weeks, eight after 3 weeks, and one after 4 weeks, making a total of 30 of the 36 treated cases which became negative. The six which continued to show hemolytic organisms were lost track of before further observations could be made.

#### *Blood Counts.*

The results of the x-ray treatment on the white blood cell count were not uniform. The counts were made at irregular intervals after meals and after a walk of several city blocks. When the lymphocytes were reduced in numbers the reduction was slight and of short duration, which would indicate that the systemic effect of the dose used was very slight.

## DISCUSSION.

The small series of cases reported here shows the possibility of materially reducing the lymphoid deposits of the nasopharynx by comparatively small doses of x-rays. Animal experiments had shown that it is possible with x-rays to induce any degree of atrophy of the lymphoid tissue without damaging other tissues. In the series of treated individuals, in all but three or four instances one treatment gave an entirely satisfactory result. In two refractory cases a second treatment was followed by the desired degree of atrophy and a clearing up of the pathological condition. It is most probable that the other few individuals who did not respond to the one treatment would have yielded on further exposure to x-rays but unfortunately the observations were discontinued before this point could be determined.

The degree of atrophy to be aimed at is a matter that experience will decide. If a reduction below the normal size and the clearing up of obvious pathological states is sufficient as has been indicated in the majority of the cases treated and observed by us, there seems to be no reason for carrying the treatment beyond this point. In view, however, of the mild nature of the treatment recommended it appears entirely safe to repeat it at suitable intervals so as to secure almost any degree of atrophy that may be desired.

Our original idea in taking up this work was that the excess of lymphoid tissue interfered with the clearing up of local infections of the pharynx. It seems probable, however, that the disappearance of infection of the tonsils and change in bacteriological flora after x-ray treatment are due to the opening up and proper drainage of the crypts which follow atrophy, rather than the actual removal of the excess lymphoid tissue.

Tonsils which have been exposed to the x-rays and not sufficiently reduced in size would in all probability be as amenable to surgical removal as before the x-ray treatment, for we have never seen any evidence of fibrosis in the lymphoid organs of animals after similar treatment. The fibroid tonsil would probably not be reduced materially by x-rays, as fibrous tissue is not appreciably affected by this agent.

## SUMMARY.

46 individuals with tonsils both hypertrophied and otherwise pathologically altered and some of whom had in addition adenoid masses and lymphoid deposits posterior to the pillars of the fauces, were given exposures to x-rays. In all but four cases the treatment was followed by marked atrophy of the tonsils and the other lymphoid deposits, attended by an opening and drainage of the tonsillar crypts. As this process progressed the previously enlarged tonsils assumed a smooth and normal appearance and the hemolytic bacteria—streptococci and staphylococci chiefly—which were often present in the affected tonsil disappeared usually within 4 weeks of the treatment.

## EXPLANATION OF PLATES.

## PLATE 103.

FIG. 1, *a* to *d*. (*a*) Condition of the tonsils before treatment; small and partly buried, with ragged surface and crypts containing exudate. In addition there were deposits behind the posterior pillars. (*b*) 1 week after x-ray treatment, showing reduction, and smoothing out of surface. (*c*) 3 weeks after treatment, with further reduction; no exudate could be expressed from tonsils. (*d*) 6 weeks after treatment. Tonsils not visible till anterior pillar was pulled back; normal in appearance. Hemolytic streptococci were present at the first examination but had disappeared by the 6th week after treatment.

## PLATE 104.

FIG. 2, *a* to *f*. (*a*) The tonsils before treatment; medium size, with deep crypts containing exudate; ragged, inflamed surface. (*b*) 8 days after treatment; some reduction. (*c*) 26 days after treatment; tonsils congested and exudate still present. (*d*) 55 days after treatment; tonsils reduced but not normal. (*e*) 1 week after a second x-ray treatment, showing further reduction, and smoothing out of surface. (*f*) 1 year and 1 month after second treatment; tonsils small and normal in appearance.

## PLATE 105.

FIG. 3, *a* to *d*. (*a*) Condition of tonsils before treatment; ragged, irregular surface, with crypts containing exudate. (*b*) 2 weeks after x-ray treatment; tonsils markedly reduced; surface irregular and congested. (*c*) 7 weeks and 5 days after treatment; tonsils normal in appearance. (*d*) 6 months after treatment; tonsils very small and normal. Hemolytic streptococci, present on first examination, disappeared after treatment.

## PLATE 106.

FIG. 4, *a* to *e*. (*a*) Tonsils before treatment; very large, ragged, and congested. Hypertrophy of lymphoid tissue behind posterior pillars. (*b*) 3 weeks after treatment; tonsils reduced in size. Lymphoid tissue behind posterior pillars entirely atrophied. (*c*) 2 months after treatment; tonsils further reduced; normal in appearance. (*d*) About 6 months after treatment; tonsils further reduced; normal surface; still some exudate on deep pressure. (*e*) 11 months after treatment; tonsils small, flat; no exudate. Hemolytic streptococci disappeared from the throat after treatment.

## PLATE 107.

FIG. 5, *a* to *e*. (*a*) Condition of tonsils before x-ray treatment; enlarged; ragged surface; deep crypts, with purulent exudate. (*b*) 7 weeks after treatment; tonsils markedly reduced; smooth, pale; no exudate on deep pressure. (*c*) 14 weeks after treatment; tonsils still further reduced; surface normal; no exudate. (*d*) 6 months after treatment; tonsils small and normal. (*e*) 1 year and 1 month after treatment; tonsils small and normal. Hemolytic streptococci disappeared from the throat by 1st week after treatment.

## PLATE 108.

FIG. 6, *a* to *e*. (*a*) Tonsils before x-ray treatment; large, ragged; crypts contain pus. Large mass of lymphoid tissue behind posterior pillars. (*b*) 2 weeks after treatment; tonsils reduced; surface smooth and clean. Mass behind pillars reduced. (*c*) 4 weeks after treatment; tonsils markedly reduced; pale and smooth; no exudate on deep pressure. (*d*) 8 weeks after treatment; small amount of exudate. (*e*) 6 months after treatment; tonsils small, normal in appearance; no exudate on deep pressure. Lymphoid tissue behind pillars practically gone. Hemolytic streptococci disappeared from throat by 2nd week after treatment.



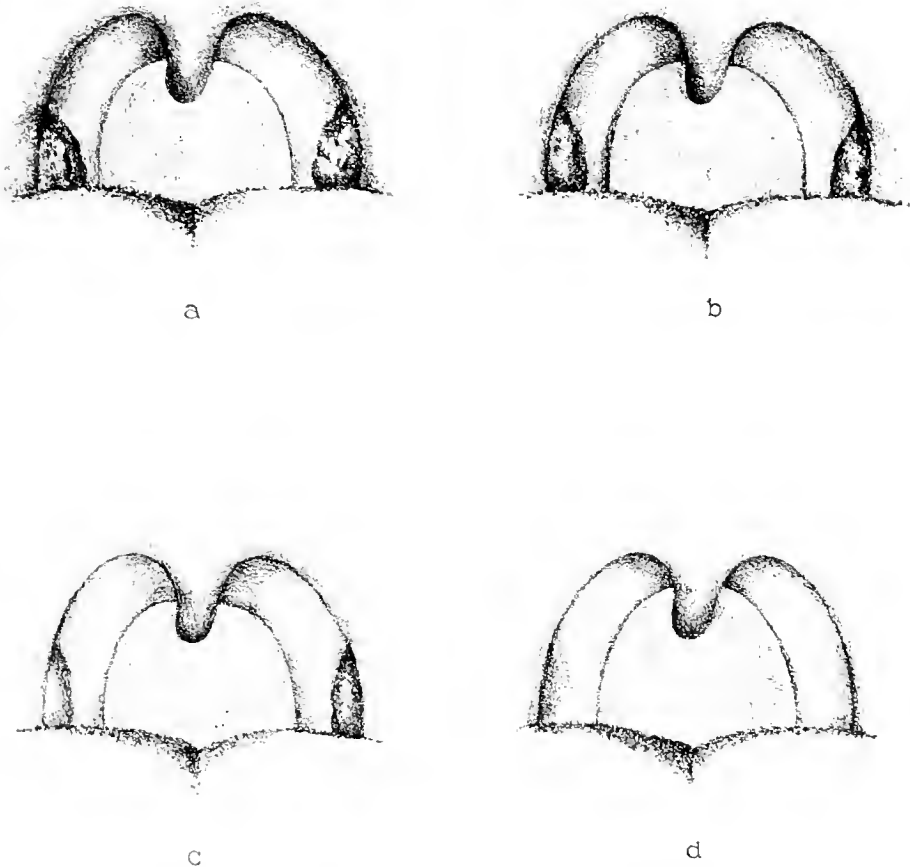
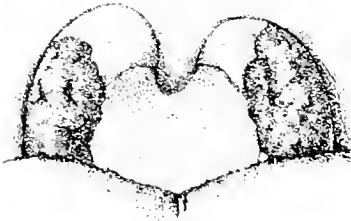


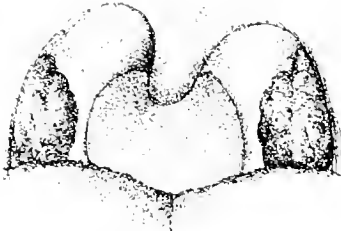
FIG. 1.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

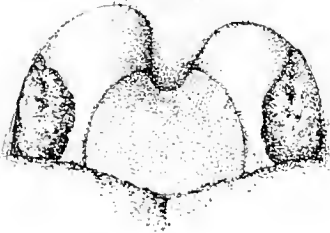




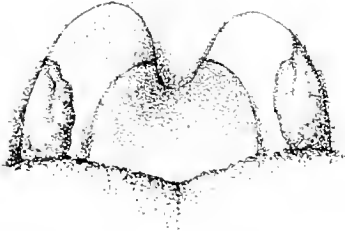
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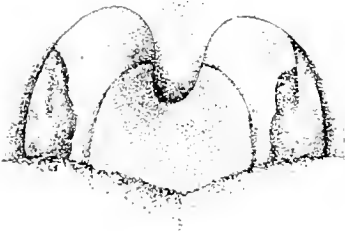
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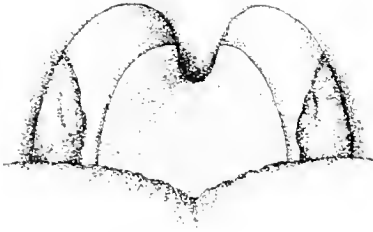
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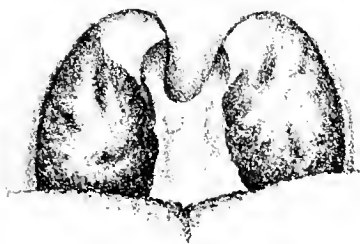


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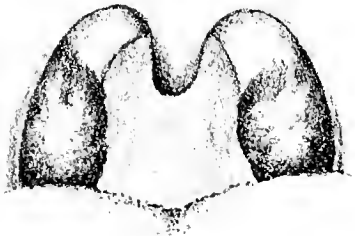
FIG. 2.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)

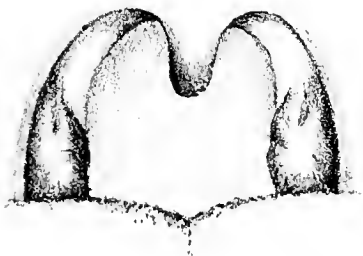




a



b



c

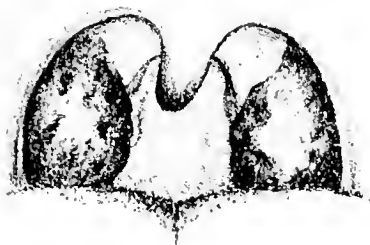


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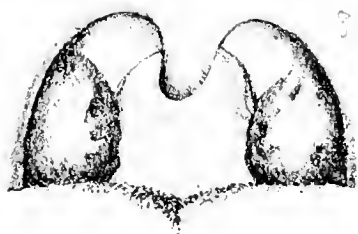
FIG. 3.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)





a



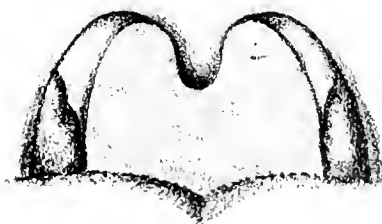
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c



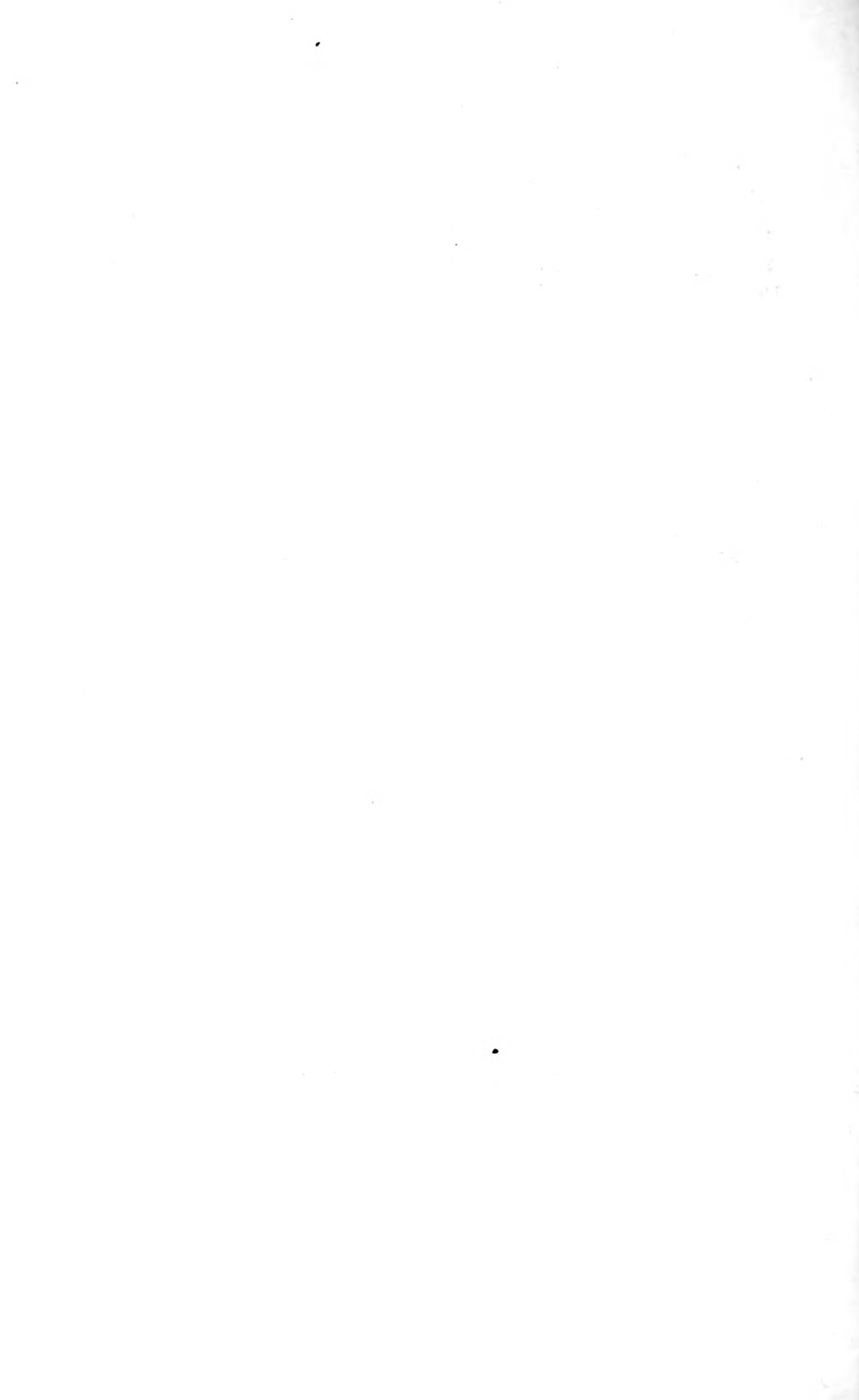
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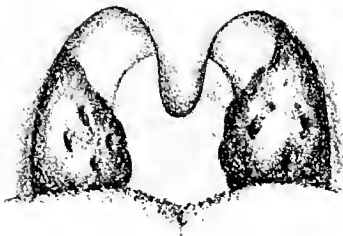
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FIG. 4.

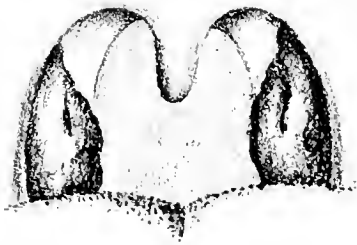
(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)







a



b



c



d



e

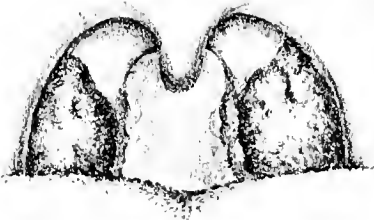
FIG. 5.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)





a



b



c



d



e

FIG. 6.

(Murphy, Witherbee, Craig, Hussey, and Sturm: Hypertrophied tonsils.)



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